Universidade do Minho
Escola de Engenharia

Roberto Andrés Gallardo Marusich

New strategies for the production of butanol and 1,3-propanediol from glycerol

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UMinho | 2013

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New strategies for the production of butanol and 1,3-propanediol from glycerol

Programa Doutoral em Bioengenharia – MIT – Portugal

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dezembro de 2013

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Título da tese

New strategies for the production of butanol and 1,3-propanediol from glycerol

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Ano de conclusão: 2013

Doutoramento em Bioengenharia (MIT Portugal)

Universidade do Minho, Dezembro de 2013

Acknowledgements

I would like to thank several people, without which the completion of this work would not have been possible.

First of all, I would like to express my sincere gratitude to my supervisors Professor Ligia Rodrigues and Professor Madalena Alves for the confidence that they placed on me and their constant support during this process.

To Professor Manuel Mota and Professor Fons Stams for their advice.

To Alcina Pereira for her help with the EGSB reactors and molecular análisis presented in Chapter 8.

To Leon Kluskens for his help with the Clostron.

To Engª Madalena Vieira for her assistance with the chromatography techniques used in this work.

To all my colleagues from the BRIDGE group for their inconditional support and for providing a pleasant working environment.

Finally, to my family who constantly motivated me to achieve my goals. I dedicate this thesis to them.

The work presented in this thesis was financially supported by a research grant SFRH/BD/42900/2008 from the Fundação para a Ciência e a Tecnologia (FCT)

Summary

The increasing demand for use of renewable resources as feedstock for the production of chemicals combined with advances in biotechnology is generating a renewed interest in the fermentative production of n-butanol and 1,3-propanediol (1,3-PDO). In this context, glycerol, a by-product of biodiesel and ethanol production, arises as a potential substrate for this purpose.

In this work, *Clostridium pasteurianum* DMS 525 was studied for butanol production using biodiesel-derived crude glycerol and pure glycerol as the carbon source. The main products obtained were butanol and 1,3-PDO. Moreover, the competitive nature of butanol and 1,3-PDO pathways was evident, and a shift to the former for higher glycerol concentrations was clearly observed. In preliminary experiments conducted in serum bottles using crude glycerol, the maximum glycerol consumption achieved was 31.83 ± 0.98 g l^{-1}, which resulted in 6.71 ± 0.42 g l^{-1} of butanol and 6.86 ± 0.51 g l^{-1} of 1,3- PDO.

To improve the butanol tolerance of *C. pasteurianum* DSM 525, random chemical mutagenesis (*N*-ethyl-*N*-nitrosourea) in solid medium was performed. Experiments resulted in the isolation of colonies growing in culture medium containing 12 g l^{-1} butanol (except in the controls). Mutant cells showed 20 % higher butanol production than the parent strain when grown in liquid medium.

Optimization of the culture medium composition and the inoculum age resulted in a glycerol consumption and a butanol titer of 45.62 ± 3.81 g $l^{-1}$and 12.4 ± 0.26 g l^{-1}, respectively. The concentration of 1,3-PDO reached 7.45 ± 0.86 g l^{-1}. In particular, iron was found to play a key role in this process. Supplementation of 3 mg l^{-1} $FeCl_2.7H_2O$ in the culture medium led to 140% increase in butanol titer.

In pH-controlled experiments, it was possible to increase glycerol consumption to a maximum of 75 g l^{-1}. Nevertheless, butanol production was around 9 g $l^{-1}$and higher concentrations of 1,3-PDO were obtained (20 g l^{-1}).

Finally, the production of 1,3-PDO was studied in continuous culture (EGSB reactors). Two pre-treatments (heat and disruption) were applied to the granular sludge in order to minimize the methane production. 1,3-PDO was always found to be the main product and only small amounts of acids were detected. Molecular biology tools (DGGE, cloning and sequencing) were used to evaluate the microbial community. A maximum 1,3-PDO yield and productivity of 0.43 g g^{-1} and 57 g $l^{-1}d^{-1}$, respectively, were achieved in the reactor operated with non-treated granular sludge (control).

The results obtained provide a deeper understanding of a complex process such as the anaerobic fermentation of glycerol using *Clostridium* spp. *C. pasteurianum* shows a great potential for butanol and 1,3-PDO production from crude glycerol. However, butanol toxicity seriously limits its titer, thus it is important to find ways to overcome this problem. On the other hand, this study proves the feasibility of 1,3-PDO production in EGSB reactors, which have the advantage of being operated under non-sterile conditions and represent a novel strategy to valorise glycerol generated as by-product in the biodiesel industry.

Sumário

A crescente procura de recursos renováveis como matéria-prima para a produção de químicos combinada com avanços na área da biotecnologia está a gerar um interesse renovado na produção de n-butanol e 1,3-propanodiol (1,3-PDO) por via fermentativa. Neste contexto, o glicerol, sub-produto da produção de biodiesel e etanol, surge como um potencial substrato para esta finalidade.

O objectivo desta tese foi estudar e otimizar a produção de butanol por *Clostridium pasteurianum* DMS 525 utilizando glicerol bruto (derivado da indústria do biodiesel) e glicerol puro como fonte de carbono. Os principais produtos obtidos foram butanol e 1,3-PDO. Adicionalmente, foi possível confirmar a natureza competitiva das vias metabólicas que resultam na produção de butanol e 1,3-PDO, tendo-se verificado claramente uma mudança para a via do butanol nas experiências em que se usaram concentrações mais elevadas de glicerol. Em experiências preliminares conduzidas em frascos utilizando glicerol bruto, o consumo máximo de glicerol foi de 31.83 ± 0.98 g l^{-1}, o que resultou em 6. 71 ± 0.42 g l^{-1} de butanol e 6.86 ± 0.51 g l^{-1} de 1,3 - PDO.

Para melhorar a tolerância ao butanol do *C. pasteurianum* DSM 525 realizaram-se ensaios de mutagénese química aleatória (*N*-etil-*N*-nitroso-ureia) em meio sólido. Estes ensaios permitiram isolar colónias capazes de crescer em meios 12 g l^{-1} butanol (com excepção dos controlos). Quando cultivadas em meio líquido, as células mutantes apresentaram uma produção de butanol cerca de 20% mais elevada do que a estirpe-mãe.

A otimização da composição do meio de cultura e da idade do inóculo resultou num consumo de glicerol e produção de butanol de 45.62 ± 3.81 g l^{-1} e 12.4 ± 0.26 g l^{-1}, respectivamente. A concentração de 1,3-PDO atingiu 7.45 ± 0.86 g l^{-1}. Estes ensaios permitiram verificar que o ferro desempenha um papel fundamental neste processo. A suplementação de 3 mg l^{-1} de $FeCl_2.7H_2O$ no meio de cultura conduziu a um aumento de cerca de 140% na produção de butanol.

Nas experiências conduzidas com controlo de pH, foi possível aumentar o consumo de glicerol até um máximo de 75 g l^{-1}. No entanto, nestas condições a produção de butanol foi de cerca de 9 g de l^{-1}, tendo-se obtido concentrações mais elevadas de 1,3-PDO (20 g l^{-1}).

Finalmente, a produção de 1,3-PDO foi estudada em cultura contínua (reactores EGSB). Para minimizar a produção de metano aplicaram-se dois pré-tratamentos (calor e ruptura) à lama granular. Verificou-se que o produto principal em todas as condições avaliadas foi 1,3-PDO tendo sido detetadas apenas pequenas quantidades de ácidos. Adicionalmente, as possíveis alterações na comunidade microbiana nestas condições foram analisadas usando ferramentas de biologia molecular (DGGE, clonagem e sequenciação). Os ensaios realizados no reator operado com lama granular não tratada (controlo) permitiram obter um rendimento e produtividade máxima de 1,3-PDO de 0.43 g g^{-1} e 57 g $l^{-1}d^{-1}$, respectivamente.

Em suma, os resultados obtidos nesta tese permitiram uma melhor compreensão do processo de fermentação anaeróbica de glicerol usando *Clostridium* spp. *C. pasteurianum* demonstrou um grande potencial para a produção de butanol e 1,3-

PDO a partir de glicerol em bruto. No entanto, a toxicidade do butanol limita seriamente o seu título. Nesse sentido, é importante encontrar formas de ultrapassar este problema. Por outro lado, esta tese demonstrou a viabilidade da produção de 1,3-PDO em reactores EGSB, sendo que estes reatores têm a vantagem de poderem ser operados sob condições não-estéreis e representam uma nova estratégia para valorizar o glicerol gerado como sub-produto na indústria do biodiesel.

Scientific output

Paper submitted in journals with peer review:

Gallardo R, Faria C, Rodrigues LR, Pereira MA, Alves M. Anaerobic granular sludge as a biocatalyst for 1,3-propanediol production from glycerol in continuous bioreactors. Bioresource Technology.

Gallardo R, Alves M, Rodrigues LR. Modulation of crude glycerol fermentation by *C. pasteurianum* DSM 525 towards the production of butanol. Biomass and Bioenergy.

Papers in preparation for submission to peer reviewed journals:

Gallardo R, Alves M, Rodrigues LR. Effect of iron concentration and inoculum age on the production of butanol from glycerol by a mutant of *C. pasteurianum* DSM 525.

Gallardo R, Alves M, Rodrigues LR. Effect of pH and N_2 sparging on the production of butanol and 1,3-propanediol by *C. pasteurianum.*

Gallardo R, Alves M, Rodrigues LR. Butanol and 1,3-propanediol production from glycerol by *Clostridium pasteurianum.* A review.

Publications in conference proceedings:

Gallardo R, Faria C, Pereira MA, Rodrigues LR, Alves MM. Production of 1,3-propanediol in EGSB reactors by open mixed cultures using glycerol as the carbon source. In 13th *World Congress on Anaerobic Digestion*. Santiago de Compostela, 2013, p. 1-4

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List of symbols and abbreviations

1,3-PDO-1,3-propanediol

3 HPA- 3-Hydroxypropionaldehyde

ADP - Adenosine diphosphate

ATP - Adenosine triphosphate

BES - 2-Bromoethanesulfonate

BLAST- Basic Local Alignment Search Tool

CoA - Coenzyme A

DGGE-Denaturing Gradient Gel Electrophoresis

DHA- Dihydroxyacetone

DHAP- Dihydroxyacetone phosphate

EGSB-Expanded Granular Sludge Bed

EMS-methanesulfonic acid ethyl ester

ENU- N-ethyl-N-nitrosourea

Fd-Ferredoxin

G3P-Glucose 3-phosphate

GC - Gas chromatography

GLY3P-Glycerol 3-phosphate

HPLC - High-performance liquid chromatography

HRT-Hydraulyc Retention Time

mRCM-modified Reinforced Clostridial Medium

NAD+/NADH - Oxidized/reduced forms of nicotinamide adenine dinucleotide

NTG-N-methyl-N′ nitro-N-nitrosoguanidine

OD – Optical density

PCR – Polymerase chain reaction

PEP- phosphoenolpyruvate

rpm - Revolutions per minute

RI-Refractive index

RNA- Ribonucleic acid

UV - Ultraviolet

VVM - volumes of gas per culture volume and minute

w – Weight

Yp/s - Yield of product (p) on substrate (s)

Chapter 1

Thesis motivation and outline

This chapter introduces the motivation for the development of this thesis focused on the production of butanol and 1,3-propanediol using glycerol as carbon source. Also, the thesis outline is presented.

1.1 Thesis motivation

In the last decades, great attention has been paid to the production of chemicals using renewable resources as a consequence of the sustained price increase of fossil fuels, limited reserves of petroleum, and environmental concerns. In particular, the production of biofuels has become an important field of research in biotechnology.

Nowadays, first-generation ethanol production is a consolidated industry in many countries and efforts are being made in order to make second-generation ethanol production economically competitive.

Even though ethanol is currently used as transportation fuel, it has been recognized that butanol, an aliphatic saturated alcohol, is substantially better for this purpose (Dürre, 2007). Furthermore, butanol possesses a broader market than ethanol since it can be used as an intermediate in chemical synthesis and as solvent for a wide variety of applications in the chemical, pharmaceutical and textile industry. Currently produced by chemical synthesis (with a few exceptions), it was estimated that the butanol production was 10–12 billion pounds (Donaldson et al., 2007), accounting for 7–8.4 billion dollar market with a projected expansion of 3% per year (Lee et al., 2008).

Nowadays, there is a renewed interest in the fermentative production of butanol. In this context, glycerol, a by-product of biodiesel and bioethanol production, is considered to be one of the best substrates for its production. The high degree of reduction of glycerol and its current great availability makes it a strong candidate for the industrial microbial production of butanol and other relevant chemicals such as 1,3-propanediol (1,3-PDO). Moreover, it has been estimated that by the year 2020, the production of glycerol will be six times more than its demand (Christoph et al., 2006).

Although fermentation of low-grade glycerol to butanol and 1,3-PDO using *Clostridium* spp. has been proven (Andrade and Vasconcelos, 2003; Jensen et al., 2012; Khanna et al., 2011; Taconi et al., 2009), there is still place for process optimization. The main goals of the current thesis are to improve the yield of butanol production from glycerol and to increase the butanol tolerance of the

producing microorganisms. It is also envisaged to explore new ways to produce 1,3-PDO.

1.2 Thesis outline

Overall, the work developed in this thesis can be divided in two major subjects; the fermentation of glycerol by *Clostridium pasteurianum* DSM 525, focused on the production of butanol, and the production of 1,3-PDO in EGSB reactors by open mixed cultures.

This document is organized in 9 chapters as follows;

Chapter 2 gives an overview of glycerol as substrate for anaerobic fermentation, and the target products, butanol and 1,3-PDO. This chapter also includes the fundamentals of solventogenic *Clostridium* spp. metabolism.

Chapter 3 presents the effect of crude glycerol concentration on the production of butanol and 1,3-PDO in batch culture (serum bottles) by *C. pasteurianum* DSM 525. Also, the issue of strain degeneration is discussed in this chapter

Chapter 4 presents the optimization of the culture medium for the production of butanol from glycerol by *C. pasteurianum* DSM 525. The effect of external butyrate and acetate addition to the culture medium is studied and the concentration of different nutrients is evaluated to identify possible limitations.

Chapter 5 presents random mutagenesis experiments conducted to improve the tolerance of *C. pasteurianum* DSM 525 to higher butanol concentrations.

Chapter 6 includes the effect of iron concentration and inoculum age on the production of butanol from glycerol using *C. pasteurianum.*

Chapter 7 presents the effect of pH and N_2 sparging on the fermentation of glycerol by *C. pasteurianum*.

Chapter 8 corresponds to the production of 1,3-PDO in EGSB reactors by open mixed cultures. The effect of inoculum pre-treatments and hydraulic retention time on the production of 1,3-PDO and microbial population is studied.

Finally, chapter 9 presents the overall conclusions and recommendations for future work.

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Chapter 2

Biological production of butanol and 1,3-propanediol from glycerol

The increasing demand for energy, sustained increase in the price of crude oil, and environmental concerns together with incentives for production of biofuels have been driving forces for the rapid growth of the worldwide biodiesel production in the last years. As the supply of oils to be converted to biodiesel is becoming increasingly competitive, the profitability of the biodiesel industry will largely depend on the ability to confer value to its by-products. Being glycerol the main by-product from biodiesel production, it is imperative to find ways to transform it into added-value products. In this context, crude glycerol has been recognized as good substrate for the production of chemicals by anaerobic fermentation. Its highly reduced nature makes it a good starting point for the production of reduced or neutral end products such as butanol and 1,3-propanediol, among others. This chapter presents an overview of glycerol as a potential substrate for anaerobic fermentation and the target products butanol and 1,3-propanediol. The fundamentals of *Clostridium* spp. metabolism are also described.

2.1 Glycerol: a promissing carbon source for anaerobic fermentation

Glycerol or glycerin is a short three-carbon chain with a hydroxyl group on each carbon. This compound can be produced from petrochemical sources but it is also generated as by-product in the production of biodiesel and ethanol, representing approximately 10% and 6% (w/w) of the production, respectively (Barbirato el at., 1998). The common way to produce biodiesel is to promote the reaction between a vegetable oil and an alcohol (usually methanol) in the presence of an alkaline catalyst, such as NaOH or KOH, in order to synthesize fatty acid alkyl esters, along with the co-product glycerol (Figure 2.1)

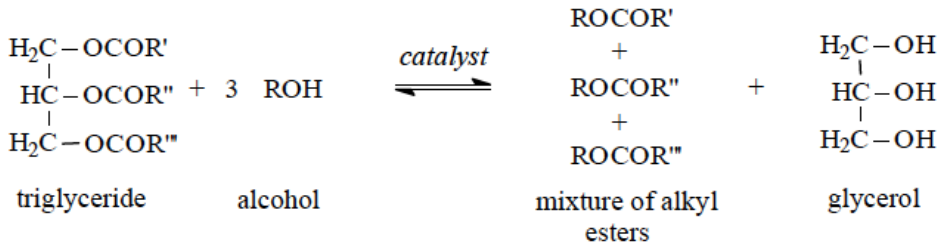

Figure 2.1.- Transesterification of a triglyceride

Although glycerol has a wide variety of applications, including ingredient or processing additive in cosmetics, toiletries, personal care, drugs and food products, this compound is typically used in a highly purified form. On the contrary, crude glycerol derived from biodiesel production possesses very low market value because of impurities, which potentially include spent catalysts, salts generated after neutralization, residual methanol, methyl esters, oil/fat, soap and free fatty acids. The chemical composition of crude glycerol mainly varies with the type of catalyst used to produce the biodiesel, the transesterification efficiency, recovery efficiency of the biodiesel, other impurities in the feedstock, and whether methanol and catalysts are recovered (Yang et al., 2012).

Even though the purification of crude glycerol is in principle affordable for large-scale biodiesel companies, the cost of this process is a limitation for small and medium producers (Pachauri and He, 2006; Dasari, 2007). Conventional techniques for purifying glycerol include distillation and ion-exchange.

Distillation is the most commonly used method and its advantages include the high-purity and high yield of the glycerol that can be obtained. However, the distillation of glycerol is an energy-intensive process due to the high heat capacity of this compound. Classical ion-exchange techniques have long been applied to glycerin purification. However, the high salt content of glycerol from biodiesel production makes the classical ion-exchange a not economically feasible approach for this application (Lancrenon and Fedders, 2008).

A key fact from the economic point of view is that for every tonne of biodiesel produced, about 100 kg of crude glycerol are generated as by-product. The fast growth that the biodiesel industry has experienced in the last years has led to a surplus of crude glycerol and therefore to a considerable fall in its price (Figure 2.2). The 2012 prices for crude glycerol in US and Europe were in the range of 6.75-8.75 cents/lb and 260-340 EUR/tonne (9.43-12.34 cents/lb), respectively (ICIS pricing 2012).

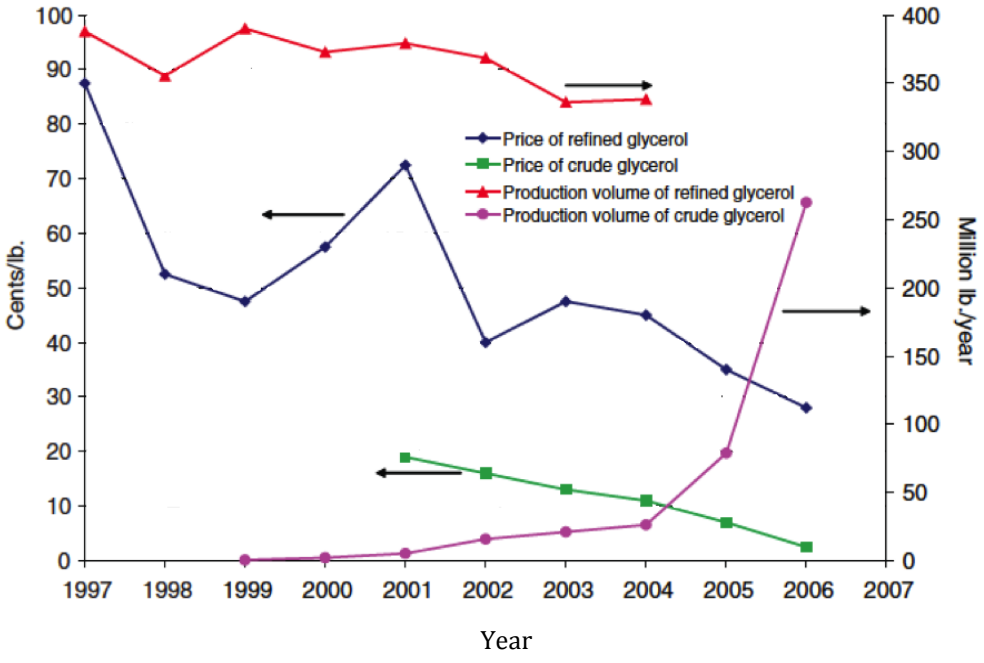

Figure 2.2. - US market prices and production volumes for refined and crude glycerol Adapted from Dasari (2007)

As the supply of oils to be converted to biodiesel is becoming increasingly competitive, the profitability of the biodiesel industry will depend on the ability to

add value to its by-products. Therefore, the conversion of crude glycerol into other useful products needs to be developed with high potential to enhance biodiesel industry and improve its economics (Choi, 2008; Johnson and Taconi, 2007).

The anaerobic microbial conversion of glycerol is particularly interesting due to the highly reduced nature of this compound, and less energy requirements in comparison with chemical transformations and aerobic biological processes. Its fermentation results in the generation of more reducing equivalents when it is converted to glycolytic intermediates (e.g. pyruvate) as compared with glucose fermentation (Yazdani and Gonzalez, 2007). This excess of reducing equivalents must be oxidized which can be accomplished by stimulating H_2 production or via various NAD(P)H consuming pathways towards reduced or neutral end products (Heyndrickx et al., 1991). In fact, glycerol can be converted into several compounds such as citric acid, lactic acid, formic acid, acetic acid, butyric acid, propionic acid, succinic acid, dihydroxyacetone (DHA), 1,3-PDO, dichloro-2-propanol (DCP), acrolein, hydrogen, butanol, ethanol, among others (Choi, 2008; Yang et al., 2012).

Several microorganisms such as *Klebsiella, Clostridium, Enterobacter* and *Citrobacter* have been reported as able to degrade glycerol (Barbirato et al., 1995; Boenigk et al., 1993; Johnson and Taconi, 2007; Kaur et al., 2012; Yang et al., 2012). *Clostridium butyricum* and *Clostridium acetobutylicum* have been the most studied microorganisms for the production of 1,3-PDO and butanol, respectively. Nevertheless, the last one can only use glycerol as co-substrate (Andrade and Vasconcelos 2003) because it cannot re-oxidize the excess of NADH generated in the cellular glycerol catabolism. The NADH-consuming 1,3-PDO pathway has shown to confer the ability of consuming glycerol to *C. acetobutylicum* (Gonzalez-Pajuelo et al., 2005).

Intensive research has been conducted to improve the biological transformation of glycerol into 1,3-PDO and butanol. However, even though interesting results have been reported, substrate and/or product inhibition are still limiting factors. Therefore, new strategies must be applied in order to make the biological production of these compounds economically competitive.

An interesting approach for the utilization of glycerol is the concept of biorefinery. Like modern crude oil refinery, the bioindustry for biofuels has a dual purpose in the economy, as it is used as a supply of energy, as well as basic chemicals (Zaborsky, 1982). Glycerol-based biorefinery was defined by Choi (2008) as the microbial fermentation processes using inexpensive and readily available glycerol as the raw material to produce fuels and chemicals. By applying this concept, a glycerol-based fermentation processes could take advantage of multiple products that can be produced from this substrate and thus, to maximize the value derived from it.

2.2 Butanol

Biofuels are an attractive alternative to existing petroleum-based fuels as they can be used as transportation fuels with little or no change to current technologies and have significant potential to improve sustainability and reduce greenhouse gas emissions. Even though gaseous biofuels, such as methane and hydrogen, can be produced microbiologically, liquid energy carriers (ethanol, butanol, among others) offer some advantages in the transportation sector (Karakashev et al., 2007) regarding storage, distribution and utilization. Among liquid biofuels, n-butanol is now receiving considerable attention.

Butanol is an aliphatic saturated alcohol having the molecular formula C_4H_9OH that, in addition to its potential use as fuel or fuel additive, can be used as an intermediate in chemical synthesis and as a solvent for a wide variety of applications in the chemical and textile industry. Specifically, this compound is used as butyl glycol ether, butyl acetate, and plasticizers; and as butyl acrylate and methacrylate esters in latex surface coating, enamels and lacquers. Furthermore, butanol is an excellent diluent for brake fluid formulations and solvent for the manufacturing of antibiotics, vitamins and hormones (Lee et al., 2008).

Compared with ethanol, currently produced from renewable resources and used as motor fuel in several countries, butanol possesses better physicochemical properties such as higher energy content, lower water absorption, better blending ability, and the possibility of being used in conventional combustion engines

without modification (Dürre, 2007). Therefore, biobutanol holds great promise for the reduction of greenhouse gas emissions generated in the transport sector.

The production of acetone, butanol and ethanol by solventogenic strains of *Clostridium* was one of the first large-scale industrial fermentation processes to be developed, and during the first part of the 20th century it ranked second in importance only surpassed by ethanol fermentation (Jones and Woods, 1986; Antoni et al., 2005). Butanol was industrially produced through the acetone–butanol–ethanol (ABE) fermentation, developed by the Russian chemist Chaim Weizmann at Manchester University, mainly from starch using *C. acetobutylicum*. However, this process was replaced by the petroleum-based butanol production due to the loss of economic competitiveness (Jones and Woods, 1986; Malaviya et al, 2011).

The increasing demand for the use of renewable resources as feedstock for the production of chemicals combined with advances in biotechnology through omics, systems and synthetic biology, metabolic engineering and innovative process developments is generating a renewed interest in the fermentative production of butanol (Fischer et al., 2008).

Despite its great potential, an efficient process based on a high performance butanol producer that can utilize inexpensive carbon substrate needs to be developed for industrial-scale butanol fermentation (Malaviya et al., 2011). The development of a glycerol-based butanol production process can add significant value to the biodiesel industry and presents excellent potential to bring economic viability to the industrial production of butanol (Malaviya et al., 2011).

Even though microorganisms such as *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharobutylicum*, *Clostridium saccharoperbutylacetonicum*, *Clostridium aurantibutyricum*, and *Clostridium tetanomorphum* are able to produce butanol along with other solvents from sugars (Jones and Woods, 1986), *Clostridium pasteurianum* naturally possesses the metabolic pathway needed for glycerol fermentation and therefore it has been studied for butanol production from this substrate (Biebl, 2001; Dabrock et al., 1992; Heyndrickx et al., 1991; Jensen et al., 2012; Khanna et al., 2011; Khanna et al., 2012; Malaviya et al., 2011; Taconi et al., 2009; Venkataramanan et al., 2012). However, butanol production by this microorgnism and other solventogenic

Clostridium spp. has been limited by relatively low butanol titers due to the toxic effects that this compound exerts to the producing organisms. As an example, wild-type strains of *C. beijerinckii* produce between 10 and 12 g l^{-1} of butanol (Blascheck et al., 2002) and the final titer of traditional butanol fermentation (*C. acetobutylicum*) cannot surpass 12-13 g l^{-1} (Jones and Woods, 1986; Papoutsakis, 2008). Final titers reported for wild-type *C. pasteurianum* spp. have been below 10 g l^{-1} with the exception of the values reported by Biebl (2001) (17 g l^{-1}). In fact, most microorganisms are unable to tolerate 1-butanol concentrations greater than 2% (v/v) (Dunlop, 2011; Knoshaug and Zhang, 2009; Li et al., 2010).

Poor solvent resistance by solventogenic clostridia and other fermenting microorganisms is the major limiting factor in the profitability of ABE production by fermentation. The toxic effect of butanol limits its concentration in the culture medium adding to the process the costs involved in solvent recovery from dilute solutions (Ezeji et al., 2010). It has been suggested that an increase in butanol titers from 12 to 19 g l^{-1}, would halve the energy required for distillation (Linden et al., 1986). Therefore, the use of genetically modified strains with improved capabilities coupled with *in situ* butanol recovery systems, such as gas stripping, seems to be the most promising alternative in the short time to overcome the current limitations of ABE fermentation. Furthermore, mutation tools specific for *Clostridium* spp. are now readily available holding a great promise for the development of more robust organisms (Heap et al., 2009).

Desired characteristics in solventogenic *Clostridium* spp. have been described by Papoutsakis (2008) and include tolerance to oxygen and possibly the ability of carrying out some aerobic metabolism; growth to higher cell densities; prolonged cell viability; direct utilization of cellulosic material; asporogenous solvent-producing strains; non-degenerative strains; solvent tolerance and improved butanol selectivity. In this context, Bennett and collaborators (2008) reported the blockage of stage II of sporulation in *C. acetobutylicum* that led cells to remain in the vegetative stage and to higher solvent titers. Recently, Mullin and Velankar (2012) reported the isolation of an aerotolerant microorganism belonging to the *Clostridium* genus able to produce butanol along with other chemicals directly from cellulose.

2.3 1,3-propanediol

1,3-propanediol (1,3-PDO), also known as a trimethylene glycol, 1.3-dihydroxypropane, and propane-1,3-diol ($C_3H_8O_2$) is an important chemical that can be used for synthesis reactions, in particular as a monomer for polycondensations (Leja et al., 2011). This product can be formulated into a wide variety of industrial products including composites, adhesives, laminates, coatings, moldings, aliphatic polyesters, co-polyesters. Examples of its applications are the synthesis of polyethylene terephthalate and polytrimethylene terephtalate, both used to make carpet fibers and comercialized by DuPont with the trade name Sorona. Furthermore, 1,3-PDO is also a solvent and can be used as antifreeze and in wood as exterior coating.

Traditional routes for the production of 1,3-PDO include chemical synthesis involving hydration of acrolein to 3-hydroxypropionaldehyde, which is then reduced to 1,3-PDO by catalytic hydrogenation. Another synthetic route consists of hydrocarbonylation of ethylene oxide by carbon monoxide and hydrogen under high pressure in the presence of catalysts and solvents. The problems with these processes are the high costs, unsatisfactory yields and the release of toxic intermediates (Choi 2008; Saxena et al., 2009) As consequence, the biological production of 1,3-PDO has been extensively studied. In this context, DuPont and Genecor International, Inc. developed a commercial biological process for the production of 1,3-PDO from glucose that makes use of a genetically modified *Escherichia coli* derived from the strain K12 (Diaz-Torres et al., 2000; Emptage et al., 2003; Laffend et al., 1997; Nair et al., 1999).

A number of microorganisms can ferment sugars to glycerol but cannot convert glycerol to 1,3-PDO. There is another group of microorganisms that are not able to convert sugars to glycerol but have the ability to ferment glycerol to 1,3-PDO and still others can ferment mixtures of glycerol and sugars to 1,3-PDO. However no wild-type strain can ferment sugars as the sole carbon source directly to 1,3-PDO (Cameron et al., 1998; Saxena et al., 2009).

The conversion of glycerol to 1,3-PDO can be accomplished by several microorganisms including bacteria of the genera *Klebsiella, Citrobacter, Enterobacter* (Barbirato et al., 1998), *Clostridium,* and *Lactobacilli* (Saxena et al.,

2009). Specifically, this process has been demonstrated in *Lactobacillus brevis, Lactobacillus buchnerii, Bacillus welchii, Citrobacter freundii, Klebsiella pneumoniae, C. pasteurianum, C. butyricum and Eenterobacter agglomerans* (da Silva et al., 2009), among others.

Even though research on the production of 1,3-PDO from glycerol has been focused mainly on *C. butyricum,* (Chatzifragkou et al., 2011; Colin et al., 2000; Giinzel et al., 1991; Gonzalez-Pajuelo et al., 2005a; Papanikolaou et al., 2004), some studies using genetically modified *C. acetobutylicum* (Gonzalez-Pajuelo et al., 2005b; Gonzalez-Pajuelo et al., 2006), *C. freundii*, *Klebsiella oxytoca* and *K. pneumoniae* have been reported (Homann et al., 1990; Menzel et al., 1997; Moon et al., 2010; Zeng et al., 1993; Zhang et al., 2006).

C. butyricum has been recognized as one of the best candidates for the production of 1,3-PDO (Saxena et al., 2009). Among its advantages, being a non-pathogenic microorganism and its ability to produce 1,3-PDO with yields close to the theoretical maximum are the most interesting (Saint-Amans et al., 1994; Gonzalez-Pajuelo et al., 2005). However, this strain is limited by a relatively low tolerance to glycerol and 1,3-PDO. It has been reported that *C. butyricum* is inhibited at 8% (wt/vol) glycerol (Biebl, 1991). In this context, *C. pasteurianum* has the advantage of tolerating glycerol concentrations up to 17% (wt/vol) (Dabrock et al., 1992). It has been also reported that in *C. butyicum* CNCM 1211 growth is completely inhibited at a 1,3-PDO concentration of 65 g l^{-1} (Colin et al., 2000). Barbirato and collaborators (1998) reported a maximum level of 1,3-PDO tolerated by *C. butyricum* CNCM 1211 of 63.4 g l^{-1}.

Another strain able to produce 1,3-PDO from glycerol that has not been widely studied is *Clostridium diolis*. Otte and collaborators (2009) reported a 1,3-PDO titer of 85 g l^{-1} using a mutant *C. diolis* DSM 15410 obtained by chemical mutagenesis, DNA shuffling and selection. This value is higher than the concentrations obtained using *C. butyricum* and represents an 80% improvement in comparison with the wild type strain.

2.4 Fundamentals of butanol and 1,3-propanediol production from glycerol

The metabolic pathway for glycerol fermentation has been observed in several different genera of bacteria belonging to the family Enterobacteriaceae and Clostridiaceae including as mentioned before species of *Klebsiella, Citrobacter, Enterobacter and Clostridium*.

Typical products generated by glycerol fermentation include 1,3-PDO, ethanol, butanol, formate, butyrate, acetate, succinate, lactate, CO_2 and H_2 (Biebl, 2001; Colin et al., 2000; Dabrock et al., 1992; Gonzalez-Pajuelo et al., 2005; Heyndrickx et al., 1991; Selembo et al., 2009; Temudo et al., 2008). In particular, in *C. pasteurianum* fermentations all the above-mentioned compounds can be detected with the exception of succinate (Figure 2.3). It is worth to note that this is the only microorganism in which the combined production of 1,3-PDO and butanol from glycerol has been reported (Biebl, 2001). On the other hand, *C. butyricum* produces 1,3-PDO, acetate, butyrate, CO_2 and H_2 but no solvents (Barbirato et al., 1998; Gonzalez-Pajuelo et al., 2006; Heyndrickx et al., 1991; Biebl, 2001). Homman and collaborators (1990) studied the glycerol fermentation of several strains of *Klebsiella* and *Citrobacter*. The authors found that *Citrobacter* strains formed 1,3-PDO and acetate and almost no by-products; while the *Klebsiella* strains produced also varying amounts of ethanol and accordingly less 1,3-PDO. Barbirato and collaborators (1998) studied the glycerol fermentation by *Citrobacter freundii* and *Klebsiella pneumoniae*. The authors reported the production of 1,3-PDO, acetate, formate and minor quantities of lactate, succinate and etanol by both microorganisms.

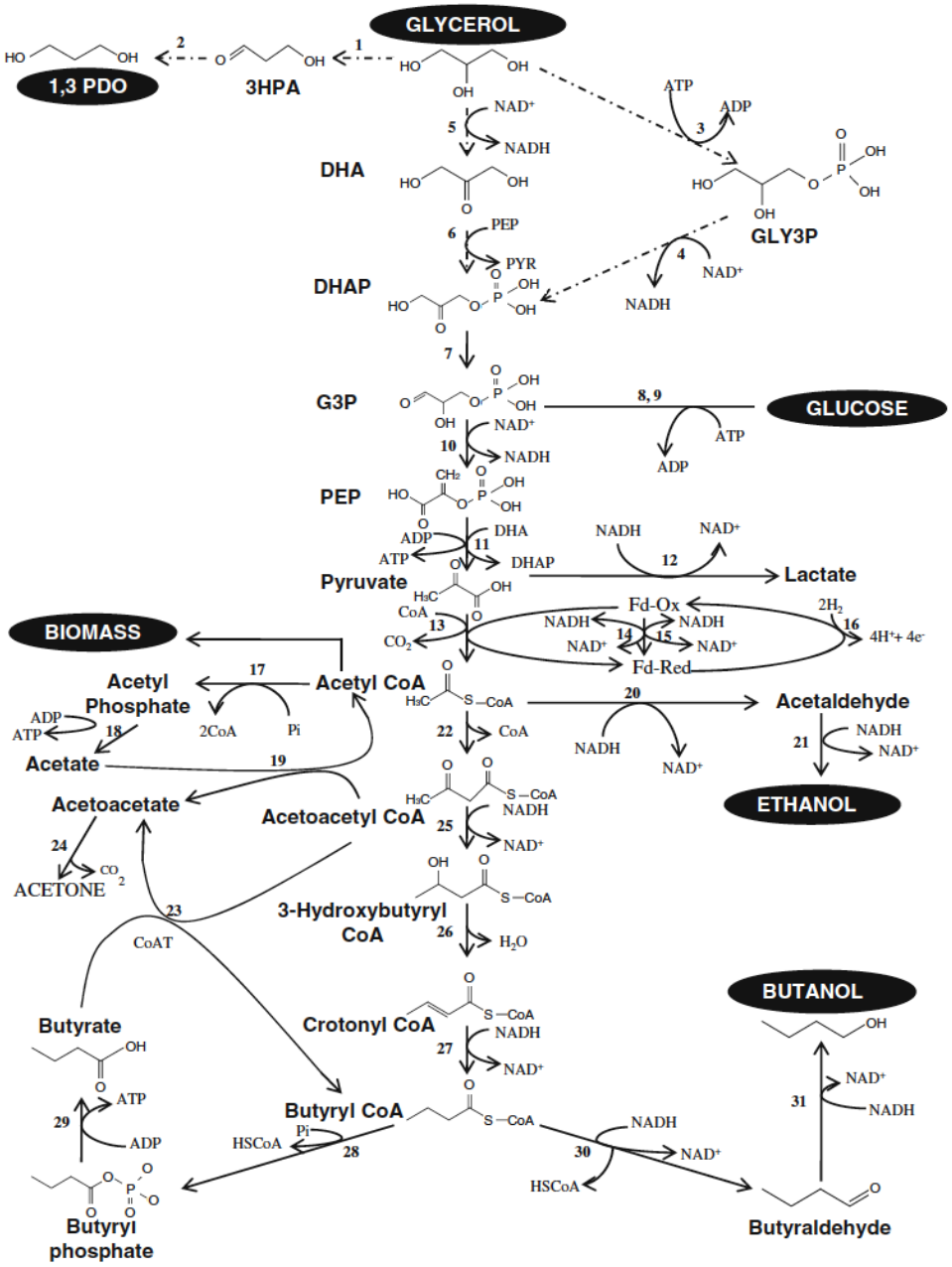

Figure 2.3. - Metabolic pathway for glycerol fermentation in *Clostridium pasteurianum*. Source: Malaviya and collaborators (2011)

C. acetobutylicum has been the model microorganism to study the metabolism of solventogenic *Clostridium* spp. The production of solvents from sugars in this microorganism is achieved in two well-defined steps. In the first step, the substrate is consumed and acids are produced (acidogenesis) which occurs in the exponential growth phase. This results in a decrease in the pH of the culture medium. As the culture enters the stationary phase, the metabolism of the cells

undergoes a shift to solvent production (solventogenesis). During this second step of the fermentation, the re-assimilation of acids occurs concomitantly with the continued consumption of carbohydrate, normally leading to an increase in the pH of the culture medium. (Jones and Woods, 1986).

Eventhough this two-step mechanism has been observed in other *Clostridium* species such as *Clostridium bejerinkii*, the regulation of the metabolic pathway in *C. pasteurianum* somehow differs, and solvents are mainly produced during exponential growth phase when glycerol is used as the carbon source. However, this only occurs after some accumulation of butyric and acetic acid (Biebl, 2001). The assimilation of acids and its conversion to solvents in *Clostridium* spp. have been seen as a detoxification process because acids are more toxic than the solvents produced (Papoutsakis, 2008) The production of hydrogen by solventogenic *Clostridium spp.* allows cells to dispose the excess of protons and electrons. This is possible due to the presence of two key enzymes, namely NADH ferredoxin oxidoreductase whose function is the transference of electrons from NADH to generate reduced ferredoxin, which in turn is used to generate molecular hydrogen by means of the action of an hydrogenase, i.e. an enzyme that enables the cell to use protons as terminal electron acceptors (Jones and Woods, 1986). The formation of acetate and butyrate are essential pathways to generate the ATP needed for biomass synthesis and other ATP-dependent processes. Although the fermentation to butanol is the energetically preferred pathway, the formation of 1,3-PDO is necessary for the reducing equivalent balance when glycerol is the carbon source. In fact, the conversion of glycerol to ethanol or butanol is a redox neutral conversion. However, glycerol is more reduced than the biomass formed and therefore, an additional electron acceptor is required (Biebl, 2001).

Even though the metabolic pathway involved in glycerol degradation in *Clostridium* spp. is well known, the environmental factors that lead these anaerobes to use one or the other metabolic route are not fully understood. Several factors have been reported to affect the product distribution in solventogenic *Clostridium* spp. such as nutrient limitation, inoculum age, pH, inoculum size, among others (Dabrock et al., 1992; Malaviya et al., 2011). Based on that, the possibility of modulating the fermentation using *Clostridium* spp. in order to

favour one or another product seems to be possible.

At molecular level, anaerobic fermentation of glycerol by *C. pasteurianum* occurs via two different concurrent metabolic pathways (Figure 2.3). In the reductive pathway, glycerol is dehydrated to form 3-Hydroxypropionaldehyde (3-HPA) by the vitamin B12-dependent glycerol dehydratase, followed by the reduction of 3-HPA to 1,3-PDO by the enzyme 1,3-PDO dehydrogenase. In the oxidative pathway, glycerol is dehydrogenated by glycerol dehydrogenase to form dihydroxyacetone (DHA), which is then phosphorylated and directed into the glycolytic pathway by dihydroxyacetone kinase. The oxidative pathway leads to the production of lactate, acetate, butyrate, ethanol, and butanol (Biebl et al., 1999; da Silva et al., 2009; Dabrock et al., 1992).

2.5 Conclusions

The rapid growth that the biodiesel industry has experienced in the last years has led to a surplus of crude glycerol and consequently to a fall in its price. Due to economic constrains, it is imperative to find ways to transform crude glycerol into value added products in order to guarantee the further development of biodiesel industry. The high degree of reduction of glycerol makes it an interesting substrate for anaerobic fermentation. Several products can be obtained from glycerol, among which butanol and 1,3-PDO are of special interest.

The production of butanol and/or 1,3-PDO from glycerol has been reported for *C. pasteurianum and C. butyricum*, among others. Nevertheless, product inhibition is the limiting factor of this process. In particular, butanol seriously limits solventogenic *Clostridium* spp. and therefore further research should be conducted in order to overcome current problems encountered and to better understand the complex metabolic regulation mechanisms present in these microorganisms.

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Chapter 3

Effect of crude glycerol concentration on the production of butanol and 1,3-propanediol by *C. pasteurianum* DSM 525

Impurities in crude glycerol can influence the microbial metabolism. It has been reported that the use of crude glycerol decreases butanol yield and results in longer fermentations in comparison with pure glycerol. Linoleic acid, potentially present in crude glycerol, has been recognized as a strong inhibitor of glycerol utilization by *C. pasteurianum* ATCC 6013. On the other hand, it has been shown that the concentration of glycerol itself has a notorious influence in the product repertoire using *C. pasteurianum* DSM 525. In this chapter, the effect of crude glycerol concentration on the production of butanol and 1,3-PDO by *C. pasteurianum* DSM 525 was studied. The results showed a marked effect on the yield of these products. The competitive nature of butanol and 1,3-PDO pathways has been evident, and a shift to the butanol pathway when using higher crude glycerol concentrations (up to 35 g l^{-1}) was clearly observed. Further increases in crude glycerol concentration did not make a difference in the outcome of the fermentation. The maximum glycerol consumption achieved was 31.83 ± 0.98 g l^{-1} which resulted in 6.71 ± 0.42 g l^{-1} of butanol and 6.86 ± 0.51 g l^{-1} of 1,3- PDO. Strain degeneration was observed in preliminary experiments; however the problem was overcome through progressive strain adaptation and selection.

3.1 Introduction

The fermentation of glycerol by *C. pasteurianum* using pure glycerol as carbon source has been reported by several authors (Ahn et al., 2011; Biebl, 2001; Dabrock et al., 1992; Heyndrickx et al., 1991; Jensen et al., 2012; Khanna et al., 2011; Khanna et al., 2012; Luers et al., 1997; Malaviya et al., 2011; Taconi et al., 2009; Venkataramanan et al., 2012). Unlike pure glycerol, biodiesel-derived crude glycerol contains impurities such as methanol, ash, free fatty acids and triglycerides that could exert a negative effect on the growth of microorganisms. However, the information reported in literature is not conclusive.

It has been suggested that among the impurities potentially present in crude glycerol, special concern needs to be taken on remaining methanol, as well as sodium or potassium salts because they are known to be inhibitory to cell growth (Choi, 2008). Taconi and collaborators (2009) reported a decrease in the average yield of butanol from 0.24 to 0.22 g g^{-1} and longer fermentation times when using crude glycerol compared with pure glycerol for *C. pasteurianum* ATCC 6013. Venkataramanan and collaborators (2012) studied the impact of impurities in biodiesel-derived crude glycerol on the fermentation by *C. pasteurianum* ATCC 6013. The authors found that linolenic acid (present in most vegetable oils used for biodiesel production), potentially present as non-reacted free fatty acid in biodiesel-derived crude glycerol, has a strong inhibitory effect on the utilization of glycerol by the bacteria. Kanna et al. (2001) using immobilized *C. pasteurianum* cells found an increase in butanol production with increasing substrate (crude glycerol) concentration and reported a maximum concentration of n-butanol (8.8 g l^{-1}) starting from 25 g l^{-1} of substrate. Further increases in crude glycerol concentration resulted in a dramatic decrease in butanol production.

On the other hand, the glycerol concentration itself, rather than the impurities that crude glycerol could contain, seems to have a strong influence in *C. pasteurianum* metabolism. In fact, Dabrock and collaborators (1992) reported a marked influence of the glycerol (pure) concentration in the product distribution using this microorganism.

The effect of crude glycerol concentration on the production of 1,3-PDO was also studied in *C. butyricum* by Papanikolaou and collaborators (2004). The authors found that for increasing glycerol concentrations in the inlet medium, the biomass

yield decreased. This decrease was attributed to the microbial metabolism being directed towards the biosynthesis of organic acids instead of biochemical anabolic reactions. By the development of analytical models, the authors showed that high inlet substrate concentrations positively affected the production of butyric acid and to a lesser extent that of acetic acid. In contrast, at increased glycerol concentrations, the relative increase of 1,3-PDO production per unit of substrate consumed was lower as compared with that of acetic and mainly butyric acid.

In this chapter the effect of crude glycerol concentration on the production of butanol and 1,3-PDO by *C. pasteurianum* DSM 525 is evaluated.

3.2 Material and methods

3.2.1 Strain maintenance and reactivation

C. pasteurianum DSM 525 was purchased from DSMZ (German collection of microorganisms and cell cultures). Freeze dried cells were reactivated in the culture medium presented in Tables 3.1 to 3.4. Since the strain is a strict anaerobe, special precautions were taken to ensure the absence of oxygen in the culture medium. Briefly, the medium was boiled and cooled down on an ice bath while nitrogen was passed through the liquid. Once cooled, the culture medium was dispensed in serum bottles that were then sealed with butyl rubber stoppers and aluminium crimps. Oxygen was removed from the headspace and the bottles were pressurized with a N_2-CO_2 gas mixture (80%-20%) using a manifold.

After autoclaving, H_2CO_3 (4 g l^{-1}) and cysteine – HCl (0.5 g l^{-1}) were added as buffer and reducing agents, respectively. Resazurin was used as redox indicator. The pH was adjusted to 7.0 whenever necessary.

The freeze-dried pellet was re-suspended with 0.5 ml of reduced medium and the mixture was transferred to a serum bottle containing the culture medium prepared as it was described above. The cells were incubated at 37 ºC until growth could be observed.

Stock cultures were prepared from this first culture and kept at room temperature. These cells were transferred to fresh medium each 1-2 months. Cells were also

maintained as glycerol (15% v/v) stocks at -80 ºC, which were prepared after growing cells to an $OD_{600\ nm}$ of 0.8 to 1.0.

Table 3.1. - Culture medium used for strain reactivation

Compound	Concentration
Glucose	40 g l^{-1}
KH_2PO_4	0.5 g l^{-1}
K_2HPO_4	0.5 g l^{-1}
Yeast extract	1 g l^{-1}
Salts + vitamins solution	50 ml l^{-1}
Trace elements solution 6	1 ml l^{-1}
Trace elements Solution 7	1 ml l^{-1}

Table 3.2. - Trace elements solution 6

Compound	Concentration
HCl	1.8 g l^{-1}
H_3BO_3	61.8 mg l^{-1}
$MnCl_2$	61.3 mg l^{-1}
$FeCl_2$	1 g l^{-1}
$CoCl_2$	64.5 mg l^{-1}
$NiCl_2$	12.9 mg l^{-1}
$ZnCl_2$	67.7 mg l^{-1}

Table 3.3. - Trace elements solution 7

Compound	Concentration
NaOH	0.4 g l^{-1}
Na_2SeO_3	17.3 mg l^{-1}
$Na2WO_4$	29.4 mg l^{-1}
$Na2MoO_4$	20.5 mg l^{-1}

Table 3.4. - Salts + vitamins solution

Compound	Concentration
NH_4Cl	24 g l^{-1}
NaCl	24 g l^{-1}
$MgCl_6.H_20$	8 g l^{-1}
$CaCl_2.2H_2O$	8.8 g l^{-1}
Biotin	20 mg l^{-1}
Nicotinamide	200 mg l^{-1}
p-aminobenzoic acid	100 mg l^{-1}
Thiamine	200 mg l^{-1}
Panthotenic acid	100 mg l^{-1}
Pyridoxamine	500 mg l^{-1}
Cyanocobalamine	100 mg l^{-1}
Rivoflavin	100 mg l^{-1}
Folate	50 mg l^{-1}
Lipoate	50 mg l^{-1}

3.2.2 Analytical methods

Acids, glycerol and 1,3-PDO were measured through high performance liquid chromatography (Aminex cation-exchange HPX-87H column) equipped with UV and RI detectors. The column was eluted isocratically with H_2SO_4 0.01 N using a flow rate of 0.7 ml min^{-1}. The column temperature was set at 60 ºC. Ethanol and butanol were measured by gas chromatography (TR-WAX capillary column) equipped with a flame ionization detector. A temperature ramp (0.5 ºC min^{-1}) was used for the column and the temperatures of the injector and detector were kept at 200 ºC and 250 ºC, respectively.

3.2.3 Experimental procedure

The strain was anaerobically cultured in 500 ml serum bottles using 200 ml working volume. Ten per cent culture volume was repeatedly transferred to increasing biodiesel-derived crude glycerol concentrations using the semi-defined culture medium presented in Table 3.5. The crude glycerol used was kindly provided by a Portuguese biodiesel producer, and its composition is given in Table 3.6. The culture medium was prepared as described in section 3.2.1. For each crude glycerol concentrations, four bottles were prepared. Samples were taken at regular time intervals from three bottles until the end of the fermentation and one bottle was used as inoculum to transfer 10% culture volume to fresh medium when cells were in a vegetative stage (12-24 h). Alternatively, cells were transferred at the end of the fermentation, thus letting them to sporulate. Crude glycerol concentrations used were 5-10-15-20-25-35-50 g l^{-1}. The initial pH was set at 6.8 ± 0.2 and cells were incubated at 37 ºC. Experiments were conducted in triplicate.

Table 3.5. - Semi-defined culture medium used to study the effect of crude glycerol concentration on the production of butanol and 1,3-PDO by *Clostridium pasteurianum* DSM 525

Compound	Concentration
Crude glycerol	up to 50 g l^{-1}
KH_2PO_4	0.5 g l^{-1}
K_2HPO_4	0.5 g l^{-1}
$MgSO_4.7H_2O$	0.2 g l^{-1}
$CaCl_2.2H_2O$	0.02 g l^{-1}
$NaHCO_3$	4 g l^{-1}
Cysteine-HCl. H_2O	0.5 g l^{-1}
NH_4Cl	3 g l^{-1}
Yeast extract	1 g l^{-1}
Resazurin	0.5 mg l^{-1}
Trace elements solution 6 (Table 3.2)	1 ml l^{-1}
Trace elements solution 7 (Table 3.3)	1 ml l^{-1}

Table 3.6. - Crude glycerol composition

Parameter	Specification	Method
Glycerol	85% min	BS 5711 T3
Ash	6% max	BS 5711 T6
Non-glycerol organic matter	2.5% max	BS 5711 T9
Moisture	8-10%	BS 5711 T8
Methanol	0.1% max	EN14110

3.3 Results and discussion

Fast growth and almost complete substrate consumption was observed for all crude glycerol concentrations tested. The main fermentation products found were acids, butanol and 1,3-PDO. Even though butanol and ethanol were initially produced in culture media containing 5 g l^{-1} crude glycerol and in one out of three bottles containing 10 g l^{-1}, the solvent producing capacity of the cells was lost and only traces of alcohols were detected in subsequent transfers. Higher production of acids, including isobutyric acid (that was not observed in normal cultures) and 1,3-PDO was detected when solvents were not present (Figure 3.1)

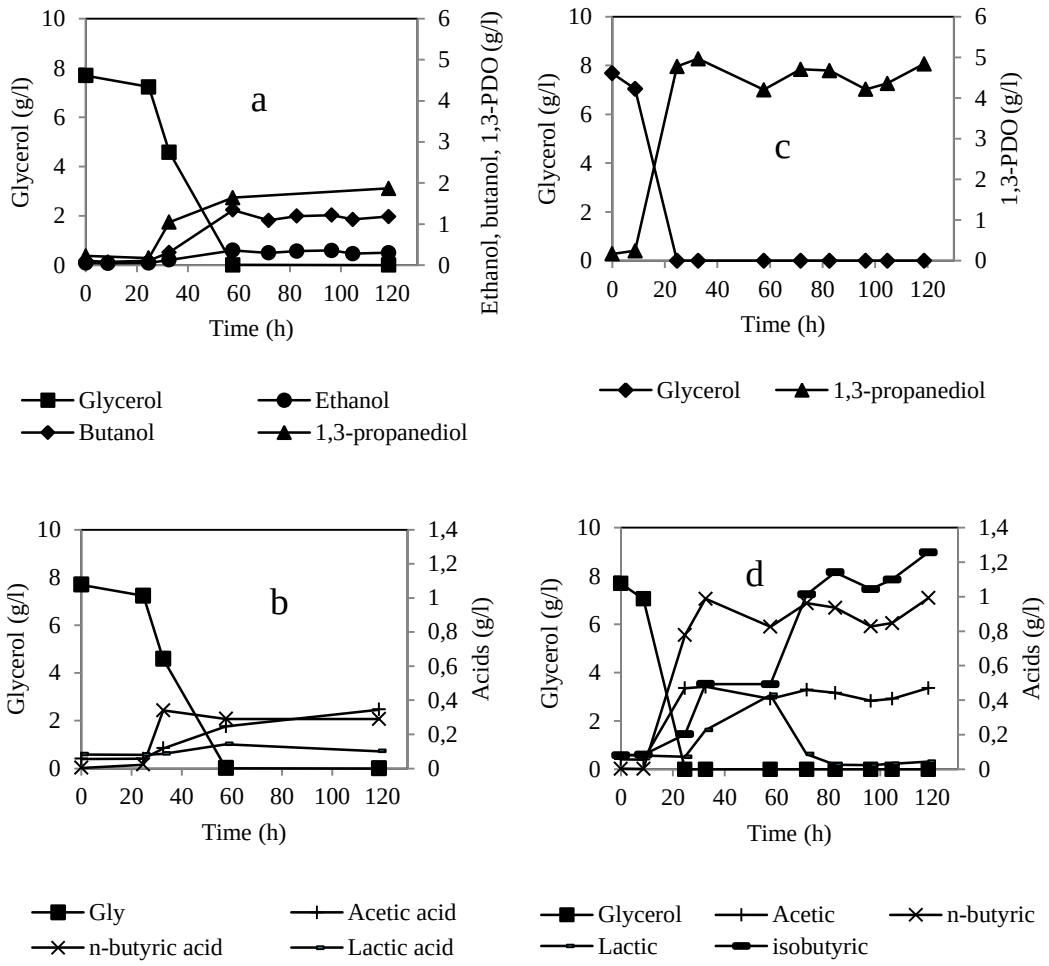

Figure 3.1. - Strain degeneration in *C. pasteurianum* DSM 525 during repeated subculturing. Production of solvents and acids from 10 g l^{-1} crude glycerol by normal (a,b) and degenerated (c,d) cells.

One of the main problems in solvent production using *Clostridium* spp., besides the toxicity of of the end product butanol, is the so-called strain degeneration. This situation is basically the loss of the solvent producing capacity of the cells during repeated subculture in batch and continuous culture and it has been reported for other solventogenic *Clostridium* species and reviewed by Jones and Woods (1986). However, little information regarding the possible occurrence of strain degeneration in *C. pasteurianum*.has been reported.

The nutrient composition of the culture medium, its pH and buffering capacity, the age and state of the cells, and the interval of time between transfers, as well as the inoculum size, all appear to affect the onset of degeneration (Jones and Woods, 1986).

Already in 1941, McCoy and Fred, reported strain degeneration for *C. acetobutylicum* in the 10-20th transfer when cells were kept in a vegetative stage. Later, Cornillot and co-workers (1997) suggested that the genes involved in solvent production in *C. acetobutylicum* reside in a plasmid whose loss leads to the strain degeneration, term they used to describe mutants that produce more acids and little or no solvents. However, this situation cannot be generalized for all *Clostridium* species. As an example, no plasmids were found in *C. beijerinckii* NCIMB 8052, which has a 6.7-Mbp single circular chromosome (Wilkinson and Young, 1995), even though degeneration also occurs in this microorganism.

Solvent production and strain degeneration have been related with the capacity of the cells to sporulate. However, it has been established that only an early step of the switch for solvent production and sporulation is activated by a shared regulatory element. As consequence, asporogenous mutants able to produce butanol have been isolated (Lemme and Frankiewicz, 1985) and obtained through directed mutagenesis (Bennet et al., 2008). The SpoOA protein has been found to positevily control the switch from acids to solvent production and the initiation of sporulation in *C. acetobutylicum* and *C. beijerinkii* by acting as a transcriptional regulator (Harris et al., 2002; Ravagnani et al., 2000).

Different strategies have been used in attempt to avoid strain degeneration. It has been reported that *C. acetobutylicum* can be subcultured in a medium containing an excess of $CaCO_3$ for more than 200 days without losing its ability to form solvents, and also that it is possible to restore initial solvent yields by increasing the size of the inoculum used (Hartmanis et al., 1986). However, Taconi and collaborators (2009) reported the loss of solvent capacity for *C. pasteurianum* ATCC 61013 (equivalent to DMS 525) while using an excess of $CaCO_3$ and this was observed even when they centrifuged and transferred the totality of the cells. Therefore, these strategies seem to be useless for *C. pasteurianum.* The addition of acetate (Chen and Blaschek, 1999 a, b) and butyrate (Yusof et al., 2010; Lee et al., 2008) to the culture medium have also shown to prevent strain degeneration and to enhance solvent production in *C. beijericnkii.*

In an attempt to restore the solvent production, and based on the information reported in the literature, those cells cultured in 25 g l^{-1} crude glycerol that did not produce solvents were transferred to culture media with the following differences:

a. 3 g l^{-1} of calcium carbonate and nitrogen in the headspace were used instead of the CO_2-$NaHCO_3$ (change in buffer system)

b. Twice the volume of inoculum (20 %)

c. 36 mM of sodium butyrate in the culture medium

Despite the new conditions used, the product profile was the same and the solvent production capacity of the cells could not be restored. This situation suggests that at least one of the enzymes involved in the conversion of sodium butyrate to butanol was not present or was inactive (butyrate-acetoacetate CoA-transferase EC 2.8.3.9; butyraldehyde dehydrogenase EC 1.2.1.57; butanol dehydrogenase EC 1.1.1) (Figure 2.3).

Since it was not possible to restore the solvent capacity of the cells, and pointing to overcome the problem of cell degeneration, new experiments were started from a stock culture using $CaCO_3$ as buffer agent instead of $NaHCO_3$-CO_2 and transferring the cells at the end of the fermentation, thus allowing them to sporulate. Even though in these new experiments strain degeneration was observed using 5 and 10 g l^{-1} crude glycerol, it was still possible to select and transfer degeneration-resistant cells, thus guaranteeing that this situation was overcome through the rest of the work.

Cells were serially transferred to culture media containing 5, 10, 20, 25, 35, and 50 g l^{-1} crude glycerol. As in the first experiments, the main products found were butanol and 1,3-PDO besides minor quantities of ethanol and acids (acetic, n-butyric, lactic, formic). Moreover, the competitive nature of butanol and 1,3-PDO pathways was evident, and a shift to the butanol pathway for higher crude glycerol concentrations (up to 35 g l^{-1}) was clearly observed (Figure 3.2). Further increases in crude glycerol concentration did not promote any improvement. This profile differs from the information reported by Dabrock and collaborators (1992). The authors studied the influence of glycerol concentration using *C. pasteurianum* DSM 525 and found that up to 8% (w/v) glycerol, the ratio ethanol/butanol/1,3-PDO did not change and that higher glycerol concentrations favoured the production of 1,3-PDO at the expense of ethanol.

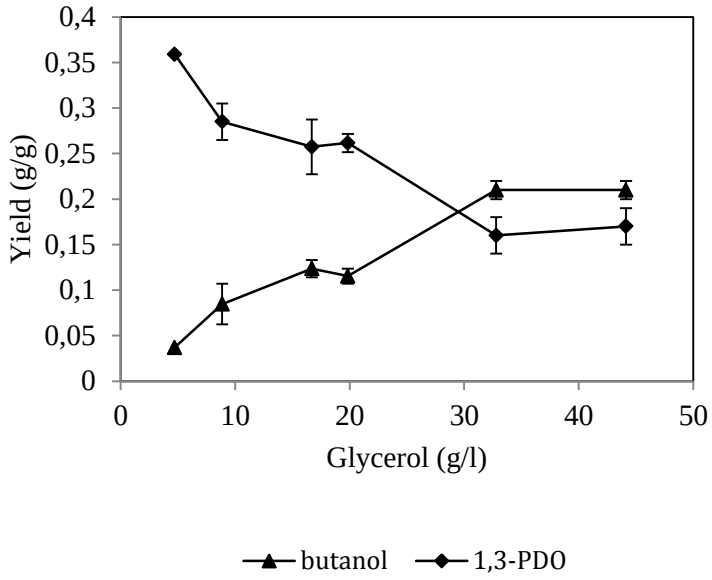

Figure 3.2. - Butanol and 1,3-PDO yield *versus* glycerol concentration in batch fermentation using crude glycerol as the carbon source. Error bars represent standard deviation of three independent experiments

The strain was able to consume up to 31.83 ± 0.98 g l^{-1} of glycerol which is slightly higher than the maximum glycerol consumption obtained by Dabrock et al. (1992) (27.6 g l^{-1}), but lower than the value reported by Biebl (2001) (63.6 g l^{-1}) while using pure glycerol in batch culture. The production of butanol and 1,3-PDO was 6.71 ± 0.42 and 6.86 ± 0.51 g l^{-1}, respectively.

Figures 3.3 and 3.4 illustrate glycerol consumption and butanol, ethanol and 1,3-PDO production along the time for cultures starting from 35 and 50 g l^{-1} of crude glycerol, respectively. A summary of the results obtained for all crude glycerol concentrations studied is presented in Table 3.7.

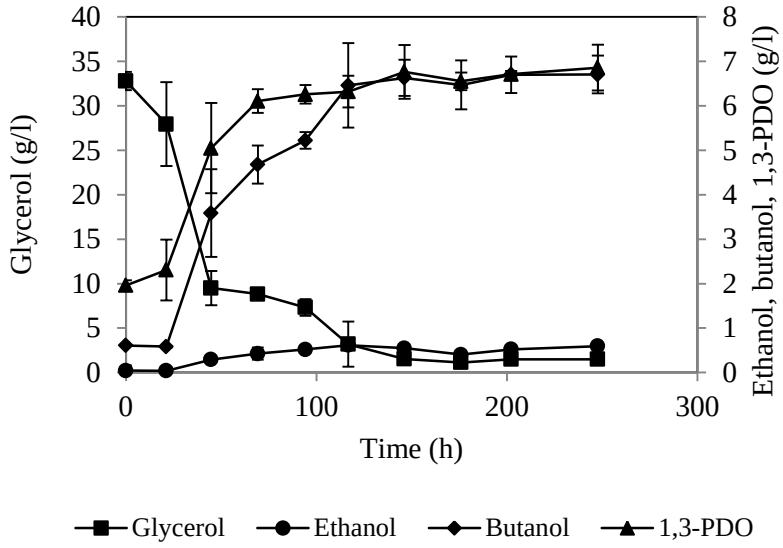

Figure 3.3. - Fermentation of 35 g l^{-1} crude glycerol by *C. pasteurianum* DSM 525. Error bars represent standard deviation of three independent experiments

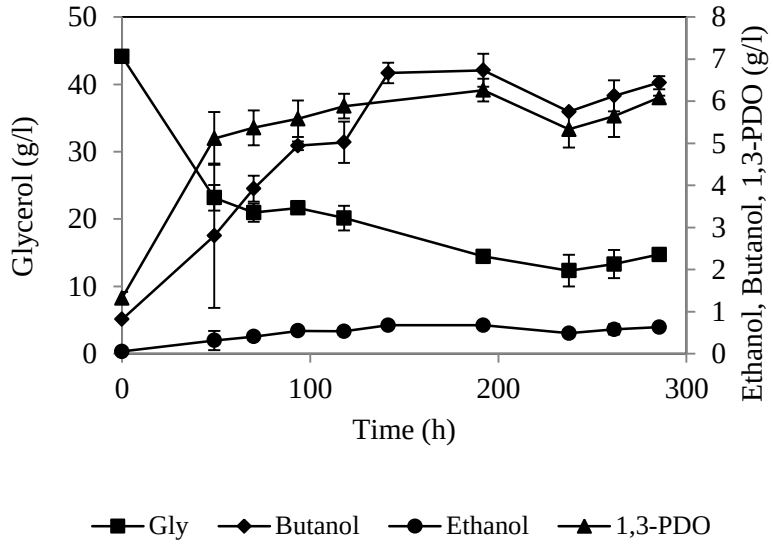

Figure 3.4. - Fermentation of 50 g l^{-1} crude glycerol by *C. pasteurianum* DSM 525. Error bars represent standard deviation of three independent experiments

Table 3.7. - Effect of crude glycerol concentration on the production of butanol and 1,3-propanediol. Results represent the average of three independent assays ± S.D.

Glycerol initial ($g\ l^{-1}$)	**Glycerol consmption ($g\ l^{-1}$)**	**Butanol production ($g\ l^{-1}$)**	**Y butanol/glycerol ($g\ g^{-1}$)**	**Ethanol production ($g\ l^{-1}$)**	**1,3-PDO production ($g\ l^{-1}$)**	**Y 1,3-PDO/glycerol ($g\ g^{-1}$)**	**Acetate production ($g\ l^{-1}$)**	**Butyrate production ($g\ l^{-1}$)**	**Lactate production ($g\ l^{-1}$)**
4.72	4.71	0.17	0.04	0.04	1.69	0.36	0.58	0.66	0.14
8.87 ± 0.08	8.83 ± 0.08	0.75 ± 0.19	0.08 ± 0.02	0.17 ± 0.01	2.52 ± 0.19	0.29 ± 0.02	0.78 ± 0.06	0.69 ± 0.06	0.27 ± 0.07
16.69 ± 1.44	16.69 ± 1.44	2.06 ± 0.11	0.12 ±0.01	0.31 ± 0.00	4.34 ± 0.15	0.26 ± 0.03	1.23 ± 0.11	1.08 ± 0.07	0.24 ± 0.13
19.84 ± 0.95	19.71 ± 1.02	2.28 ± 0.09	0.12 ± 0.01	0.26 ± 0.01	5.16 ± 0.22	0.26 ± 0.01	1.38 ± 0.04	1.50 ± 0.08	0.56 ± 0.07
32.80 ± 1.02	31.30 ± 0.52	6.60 ± 0.15	0.21 ± 0.01	0.57 ± 0.04	4.94 ± 0.52	0.16 ± 0.02	0.78 ± 0.10	0.38 ± 0.12	0.58 ± 0.28
44.13 ± 1.02	29.55 ± 1.33	6.12 ± 0.15	0.21 ± 0.01	0.64 ± 0.02	4.98 ± 0.34	0.17 ± 0.02	0.71 ± 0.05	0.21 ± 0.02	1.06 ± 0.39

It is important to stress that it is unlikely to have methanol concentrations in the culture medium high enough to exert a severe inhibition of the cell metabolism. As an example, it was reported that 40 g l^{-1} methanol caused 61% inhibition in cell growth in *C. acetobutylicum* (Hermann et al., 1985). The maximun concentration of methanol in the crude glycerol used in this work was 0.1%, which could result in a maximum of 0.5 g l^{-1} of this compound for a medium containing 50 g l^{-1} crude glycerol. Furthermore, economic constraints are driving forces for biodiesel producers to recycle methanol that is used in great excess in the process. Methanol recovery can make the biodiesel production process more efficient from both economical and environmental point of view, as it can save the input costs for the process as well as helps to maintain the specific standard. Indeed, it has been reported that some biodiesel manufacturers are now using distillation columns and flash evaporation for methanol recovery (Dhar and Kirtania, 2009).

The fact that the strain was not able to consume more than 32 g l^{-1} of glycerol is likely to be related with nutrient limitation, product inhibition, pH and/or other physiological parameters. Three studies have been reported in literature in which *C. pateurianum* DSM 525 (or its equivalent ATCC 6013) and glycerol concentrations higher than 35 g l^{-1} were used in batch culture. Dabrock and collaborators (1992) studied the effect of glycerol concentration on product distribution using a minimal medium. The authors found that the strain was able to ferment maximally 300 mM (27.6 g l^{-1}). The concentrations of 1,3-PDO, ethanol and butanol reported using 17% (w/v) glycerol were 132 mM (10.04 g l^{-1}), 30 mM (1.38 g l^{-1}) and 45 mM (3.33 g l^{-1}), respectively. Furthermore, the authors found that in continuous culture at pH values below 5.5, *C. pasteurianum* growth and substrate turnover already started to decrease and that at pH values below 4.8 the steady-state conditions could not be obtained anymore.

In the experiments herein conducted in batch culture the minimum pH value measured was 4.8, however usual values were over 5.0.

Biebl (2001) studied the effect of pH on glycerol fermentation by *C. pasteurianum* DSM 525 in a pH-controlled reactor over a range of 4.5 to 7.5. Similarly to Dabrock and collaborators (1992), the author found that at pH 4.5 only part of the substrate was used after prolonged fermentation time. The optimal pH for butanol production was 6.0. At this pH, around 50 g l^{-1} of glycerol were

consumed and 11 g l^{-1} of butanol were produced. A maximum glycerol consumption of 63.6 g l^{-1} with the production of 14 g l^{-1} of butanol was obtained when using 114.6 g l^{-1} initial glycerol concentration.

Malaviya and collaborators (2011) developed a hyper-producing mutant of *C. pasteurianum* by random chemical mutagenesis. In preliminary experiments they compared the mutant strain with the parent strain in flasks cultures using 80 g l^{-1} glycerol and reported an average butanol production of 10.8 g l^{-1} and 7.7 g l^{-1}, respectively. No information on the glycerol consumption was provided in these experiments. Interestingly, when the same experiment was conducted in a pH-controlled reactor (pH above 4.5 or 4.8) the butanol concentrations obtained increased to 10 g l^{-1} and 13.7 g l^{-1} for the parent and mutant strain, respectively, and a glycerol consumption of about 42 g l^{-1} was found for the former. The authors studied several parameters using the mutant strain, such as initial cell density, inoculum age and initial pH. In these experiments, conducted in serum bottles, they reported a maximum butanol production of 9.4 ± 0.02 g l^{-1}. Again, when the mutant strain was grown in a pH-controlled reactor (pH above 4.5 or 4.8), the butanol production increased to 14.2 ± 0.12 and 15.5 ± 0.09. It is worth to note that this is almost the same value obtained by Biebl (2001) using a wild-type *C. pasteurianum* DSM 525.

In summary, significant differences in glycerol consumption and butanol production have been reported in the literature, reflecting the complexity of the regulatory mechanisms present in this strain. These differences can be partially explained by the culture media composition but undoubtedly other variables are also involved in the process. Considerable differences in the fermentation outcome exist between cultures conducted in serum bottles and in reactors. Overall, the differences between these culture systems are the agitation, pH control and the partial pressure of hydrogen and carbon dioxide. Reactors are usually operated as an open system in which an inhert gas, such as nitrogen, is passed through the liquid (at least until cells start to produce their own gases) in order to maintain anaerobic conditions. On the other hand, serum bottles are a closed system in which gas production leads to an increase in the partial pressure of the compounds generated in the headspace. *C. pasteurianum* produces high amounts of gas (hydrogen and carbon dioxide) during the fermentation. Eventually, the concentration of these compounds in the liquid phase can reach

inhibitory concentrations affecting the cell metabolism. As an example, the inhibition of hydrogenases could lead to a decrease in the pH of the culture. Adding this to the fact that in serum bottles the pH cannot be controlled, the situation can lead to fermentation stoppage, resulting in incomplete susbstrate consumption and butanol titers lower than inhibitory values. Indeed, butanol titers reported in literature for *C. pasteurianum* fermentation in serum bottles do not surpass 9.4 g l^{-1} (Malaviya et al., 2011)

Even though the above-mentioned studies give some insights about important parameters in the fermentation of glycerol by *C. pasteurianum*, the information is limited and at this point it is not possible to conclude about the situation of incomplete substrate consumption obtained in experiments herein conducted.

3.4 Conclusions

Similar to results reported for *C. acetobutylicum* and *C. beijerinkii*, strain degeneration was also observed in *C. pasteurianum* DSM 525 during repeated subculturing. Furthermore, it was not possible to restore the solvent producing capacity of cells. The situation was overcome by using culture conditions reported in literature. In particular, the use of calcium carbonate as buffer agent and the transference of sporulated cells allowed subculturing for 8 generations without any sign of degeneration.

Crude glycerol concentration showed to have a marked effect on *C. pasteurianum* metabolism. Even though 1,3-PDO was the main product at low glycerol concentrations, a shift to the butanol pathway was observed as the concentration of glycerol was increased up to 35 g l^{-1} what indeed is a positive fact for the industrial production of butanol.

The maximum glycerol consumption was obtained at 35 g l^{-1} crude glycerol concentration. At a 50 g l^{-1} crude glycerol concentration the consumption and butanol and 1,3-PDO production did not change, thus suggesting that the culture was limited either by the lack of some nutrient or product inhibition. However, other variables such as inoculum age and the culture pH cannot be discarded.

3.5 References

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Chapter 4

Culture medium optimization for the production of butanol by *C. pasteurianum* DSM 525

The effect of butyrate and acetate supplementation in the culture medium on the production of butanol and 1,3-PDO by *C. pasteurianum* DSM 525 was studied. Both cases resulted in a higher butanol production at the expense of 1,3-PDO. Furthermore, butyrate supplementation decreased the fermentation time. The maximum butanol titer of 9.75 ± 0.45 g l^{-1} was obtained using a culture medium with 50 g l^{-1} crude glycerol and 36 mM sodium butyrate. The 1,3-PDO yield and titer were significantly lower than the controls, reaching 0.07 ± 0.008 g g^{-1} and 3.04 ± 0.05 g l^{-1}, respectively. No appreciable differences were found in glycerol consumption.

Subsequently, the culture medium composition was evaluated to identify possible nutrient limitations. Even though glycerol consumption could not be increased, a simultaneous increase in $NHCl_4$ (from 3 to 5 g l^{-1}) and $FeCl_2$ (from 1 to 11 mg l^{-1}) showed a positive effect in butanol production (butanol yield on glycerol increased from 0.19 ± 0.01 to 0.27 ± 0.01 g g^{-1}, whereas the 1,3-PDO yield decreased from 0.15 ± 0.02 to 0.06 ± 0.01 g g^{-1}). Under these conditions the concentration of butanol and 1,3-PDO reached 9 ± 0.08 g l^{-1} and 1.78 ± 0.24 g l^{-1}, respectively.

The results suggest that there are factors other than butanol inhibition and nutrient limitation that affect glycerol consumption. On the other hand, it was demonstrated the possibility of modulating the fermentation towards the production of butanol by supplementing the culture medium with sodium butyrate or by adjusting the concentration of key nutrients.

4.1 Introduction

Despite the importance of the culture medium composition as a key factor in the outcome of the fermentation, most of the work conducted with *C. pasteurianum* that has been reported does not evaluate this issue. Furthermore, standard medium formulations commonly used to grow solventogenic clostridia have not dramatically changed along the time (Heluane et al., 2011).

In this chapter, the supplementation of butyrate and acetate to the culture medium is studied. Also, the concentration of the culture medium components is evaluated to identify any possible limitation and thus to optimize the production of butanol.

4.1.1 Effect of butyrate and acetate

As it was pointed out in Chapter 2, solvent production by *C. acetobutylicum* and *C. beijerinkii* is characterized by two well-defined steps, namely acidogenesis and solventogenesis.

The metabolic pathways involved in the production of acetate and butyrate in solventogenic clostridia are well established (Gheshlaghi et al, 2009; Malaviya et al., 2011). The production of acetate occurs via pyruvate - Acetyl CoA - Acetyl phosphate - acetate, whereas butyrate is produced via pyruvate - Acetyl CoA - 3-hydroxybutyryl CoA - Crotonyl CoA - butyryl CoA - Butyryl phosphate - Butyrate (Figure 2.3).

When acids are re-assimilated, acetate and butyrate are converted to Acetyl CoA and Butyryl CoA, respectively. Eventually, both intermediates can be directed to the production of solvents. It has been suggested that the uptake of acids by cells acts as a detoxification process in response to an unfavourable environment, since the solvents are less toxic than the acids produced (Costa, 1981; Nicolau et al., 2010). It is worth to note that only the undissociated form of acids can enter the cell and therefore acid reassimilation can only occurs at low pH values, which is one of the factors reported as involved in the onset of solvetogenesis in *C. acetobutylicum*.

Even though the conversion of butyrate to butanol does not occur in the absence of a reduced carbon source such as glucose or glycerol (required for ATP and

NADH generation through glycolysis), it has been reported that the supplementation of the culture medium with this compound (and also with acetate) enhances solvent production in some strains.

Hartmanis and collaborators (1984), by using ^{13}C NMR (nuclear magnetic resonance), found that externally added butyrate was taken up from the culture medium by *C. acetobutylicum* ATCC 824 and quantitatively converted to butanol without accumulation of intermediates. Chen and Blaschek (1999a, 1999b) reported that the addition of acetate to the culture medium increases and stabilizes solvent production and also increases glucose utilization by *C. beijerinckii* NCIMB 8052 and *C. beijerinckii* BA101 (solvent-hyper producing mutant derived from *C. beijerinckii* NCIMB 8052). Lee and collaborators (2008) also found that the addition of acetate and butyrate enhances solvent production by *C. beijerinckii* NCIMB 8052 and affects the ratio of acetone/butanol. The authors reported an increased butanol production during the early exponential phase when using butyrate. Furthermore, the addition of this compound to the culture medium was found to prevent strain degeneration during an extended sub-culturing and continuous culture.

Even though it has been demonstrated that it is possible to obtain higher butanol concentrations when butyrate or acetate is supplemented to the culture medium under certain conditions, the practical limit is still the butanol tolerance of the microorganisms. Thus, if the cells are producing butanol at inhibitory levels, the supplementation of these compounds should not result in an improvement in terms of solvent production unless functions associated with the uptake of acids and its conversion to solvents are not affected. However, it has been reported that the accumulation of butanol affects the cell membrane, which ultimately results in the loss of essential functions.

On the other hand, if cells are producing butanol at sub-inhibitory concentrations, a higher butanol titer can be expected when butyrate or acetate is added to the culture medium. As it was pointed out by Datta and Zeikus (1985), higher butanol titers obtained at the expense of butyrate must be accompanied with a change in the electron flow towards the production of solvents. In the case of *C. pasteurianum* growing on glycerol, an increase in butanol production from externally added butyrate should be accompanied in a decrease in the production

of other reduced products such as of 1,3-PDO, ethanol, lactate or H_2. This situation can be result of a higher activity of enzymes involved in the production of solvents from acetate or butyrate. Indeed, Chen and Blanchek (1999a) reported a higher CoA transferase activity and higher acetate kinase- and butyrate kinase specific activity in *C. beijerinckii* NCIMB 8052 grown in a culture medium supplemented with sodium acetate. Husemann and Papoutsakis (1989) found that the levels of NAD-dependent and NADP-dependent butyraldehyde dehydrogenase, which catalyzes the conversion of butyryl-CoA to butyraldehyde (precursor of butanol), were induced more strongly in the presence of CO and butyric acid.. Datta and Zeikus (1985) demonstrated that metabolic modulation by CO (inhibitor of hydrogenase) was particularly effective when acetate or butyrate at 5 g l^{-1} were added to the fermentation as electron sinks.

4.1.2 Nutrient limitation

Residual concentrations of carbon source at the end of the fermentation usually are related with the lack of some nutrient in the culture medium or product inhibition. Nutrient limitation in batch culture may be defined as a situation in which cellular growth is restricted due to the exhaustion of an essential nutrient. In some cases, nutrient limitation is a useful condition to improve a desired characteristic in a microbial process. For example, phosphate proved to be a suitable and reliable growth-limiting factor to ensure a high substrate turnover with a high yield of solvents in *C. acetobutylicum* (Dabrock, 1992). However, in other cases, nutrient limitation can lead to the stoppage of the fermentation and therefore, the identification of the nutrient that is causing the problem is required.

A common and simple technique to get preliminary information about possible nutrient limitations is to increase the concentration of individual compounds or group of compounds in the culture medium through independent experiments. When an increase in carbon source degradation is achived, the composition of the culture medium can be adjusted thus overcoming the nutrient limitation. However, it is worth to mention that by using this technique, the interaction between nutrients is not taken into account and therefore in some cases an experimental design will be more suitable to evaluate possible nutrient limitations.

4.2 Material and methods

The effect of butyrate on the fermentation of crude glycerol by *C. pasteurianum* DSM 525 was evaluated in culture media containing 5 to 50 g l^{-1} crude glycerol by using sodium butyrate in a concentration of 36 mM. The supplementation of sodium acetate in the same concentration was also evaluated in culture media containing 50 g l^{-1} crude glycerol. The experiments were conducted in triplicate and in parallel with those ones presented in Chapter 3 (controls) and the same materials and methods were used.

In order to obtain preliminary information about possible nutrient limitations and aiming to improve glycerol consumption and butanol production, the concentration of different nutrients (NH_4Cl, $CaCO_3$, $FeCl_2$, microelements, salts, yeast extract and biotin) was evaluated. The experiments were conducted in duplicate in 160 ml serum bottles containing 60 ml of the following culture medium, used as control (Table 4.1).

Table 4.1. - Culture medium used as control for the identification of nutrient limitation

Compound	**Concentration**
Crude glycerol	50 g l^{-1}
KH_2PO_4	0.5 g l^{-1}
K_2HPO_4	0.5 g l^{-1}
$MgSO_4.7H_2O$	0.2 g l^{-1}
$CaCl_2.2.H_2O$	0.02 g l^{-1}
$CaCO_3$	3 g l^{-1}
Cysteine-$HCl.H_2O$	0.5 g l^{-1}
NH_4Cl	3 g l^{-1}
Yeast extract	1 g l^{-1}
Resazurin	0.5 mg l^{-1}
Trace elements solution 6 (Table 3.2)	1 ml l^{-1}
Trace elements solution 7 (Table 3.3)	1 ml l^{-1}

The bottles were inoculated with 6 ml of an exponential culture of *C. pasteurianum* and incubated at 37 ºC. Samples were taken immediately after inoculation and at regular time intervals, until 137 hours. Products were measured as indicated in section 3.2.2. The different combinations of nutrient concentrations studied are presented in Table 4.2.

Table 4.2. - Nutrient combinations used to identify possible limitations

Exp.	Crude glycerol ($g\ l^{-1}$)	NH_4Cl ($g\ l^{-1}$)	$CaCO_3$ ($g\ l^{-1}$)	$C_4H_7NaO_2$ (mM)	C_2H_3NaO2 (mM)	Yeast extract ($g\ l^{-1}$)	Biotin ($mg\ l^{-1}$)	p-aminobenzoic acid ($mg\ l^{-1}$)	$FeCl_2$ ($mg\ l^{-1}$)	Salts[a]	Microelements[b]
1	35	3	3	0	0	1	0	0	1	x 1	x 1
2	50	3	3	0	0	1	0	0	1	x 1	x 1
3	50	5	5	0	0	1	0	0	1	x 1	x 1
4	50	5	3	0	0	1	0	0	11	x 1	x 1
5	50	5	3	0	0	1	0	0	11	x 1	x 3
6	50	5	5	0	0	2	0	0	11	x 1	x 3
7	50	5	5	0	0	1	0	0	11	x 1.5	x 3
8	50	5	10	0	0	1	0	0	11	x1	x 3
9	50	5	1.5	0	0	1	0	0	11	x1	x 3
10	50	5	3	36	0	1	0.01	1	11	x1	x 3

[a] Corresponds to KH_2PO_4, K_2HPO_4, and $MgSO_4.7H_2O$

[b] Corresponds to trace elements solution 6 and solution 7

4.3 Results and discussion

4.3.1 Effect of acetate and butyrate supplementation to the culture medium

Overall, the supplementation of the culture medium with 36 mM sodium butyrate resulted in higher butanol titers, thus demonstrating that glycerol consumption in the control cultures was not limited by butanol inhibition. As pointed in section 4.1, it is important to stress that a higher butanol titer obtained as a result of butyrate addition does not necessarily means a higher butanol on glycerol yield since butyrate can be directly converted into butanol via butyryl-CoA – butyraldehyde – butanol. However, butyric acid was significantly consumed only in cultures with 35 and 50 g l^{-1} initial crude glycerol concentrations. Therefore, higher butanol titers obtained at lower glycerol concentrations (< 35 g l^{-1}) cannot be attributed to the consumption of the exernally added butyrate but to an enhancement effect. For 50 g l^{-1} crude glycerol cultures, the final butanol concentration was 9.75 ± 0.45 g l^{-1} *versus* 6.73 ± 0.39 g l^{-1} in the control. The concentration of 1,3-PDO reached 3.04 ± 0.05 g l^{-1} *versus* 6.26 ± 0.27 g l^{-1}. The glycerol consumption was not significantly affected, being 27.54 ± 2.54 g l^{-1} (Figure 4.1).

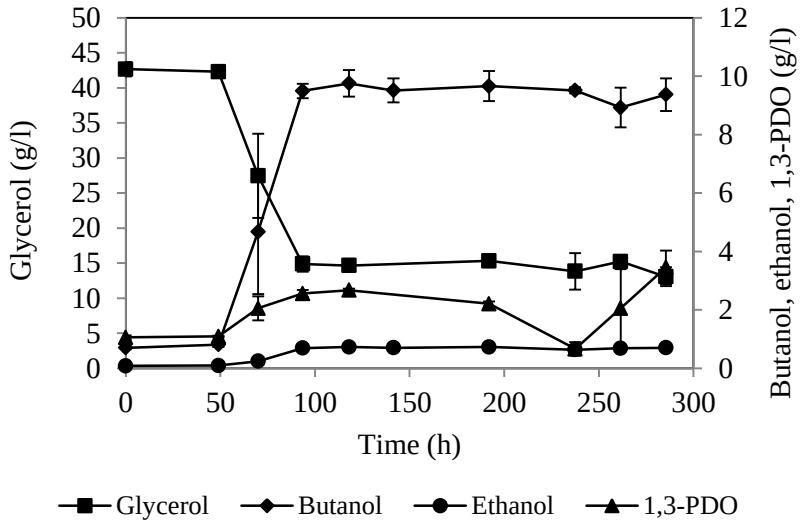

Figure 4.1. - Glycerol fermentation by *C. pasteurianum* DSM 525 using 50 g l^{-1} crude glycerol and 36 mM sodium butyrate. Error bars represent standard deviation of three independent experiments

The following table shows the initial and final concentrations of butyric acid for the fermentation presented in Figure 4.1 and the control (Figure 3.4).

Table 4.3. - Butyric acid concentrations in the cultures where butanol supplementation was used and the control. Results represent the average of three independent experiments ± S.D.

	Butyric acid (g l^{-1})	
	Culture supplemented with butyrate	**Control culture**
Initial	3 ± 0.02	0
Final	0.31 ± 0.07	0.21 ± 0.02

As it is shown in Table 4.3, the culture where 36 mM of sodium butyrate was supplemented, 2.69 ± 0.07 g l^{-1} of butyric acid was consumed. On the contrary, the control culture produced 0.21 ± 0.02 g l^{-1} of this compound. If we consider an equimolar conversion of butyrate to butanol, and that all external butyrate was converted to butanol, the amount generated by this way would be 2.26 g l^{-1}, which is almost the difference observed between the butanol production in the culture where external butyrate was added and the control (3.02 ± 0.60 g l^{-1}).

It is interesting to notice the difference in the fermentation time between the cultures supplemented with sodium butyrate and the controls. In the last case, it can be observed that the maximum butanol production and glycerol consumption was achieved in about 150 hours after inoculation in cultures starting with 35 and 50 g l^{-1} crude glycerol (Figures 3.3. and 3.4, respectively). On the other hand, the same was achieved in about 100 hours in the culture started with 50 g l^{-1} crude glycerol supplemented with 36 mM sodium butyrate (Figure 4.1). Even though a longer lag phase was observed in the culture supplemented with butyrate, the difference in the total fermentation time and the higher butanol titer resulted in more than twice the butanol volumetric productivity.

The fifty hours in which butanol was produced clearly coincides with the consumption of butyric acid, which started after a marked drop in the pH from 6.99 ± 0.03 to 5.46± 0.015 (Figure 4.2). After butyric acid was consumed, butanol reached its maximum concentration and the pH increased to a maximun of 5.90 ± 0.17.

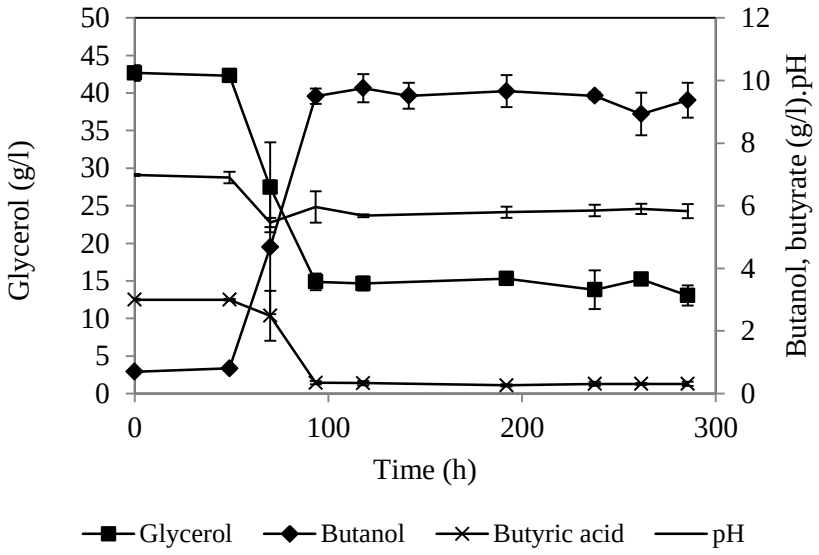

Figure 4.2. - Butyric acid consumption and pH behaviour during butanol production from glycerol by *C. pasteurianum* DSM 525. In this experiment 50 g l^{-1} crude glycerol and 36 mM sodium butyrate were used. Error bars represent standard deviation of three independent experiments

Cultures containing 36 mM sodium butyrate showed the same yield behaviour of those without external butyrate addition, i.e. an increase in the butanol yield at the expense of 1,3-PDO as the crude glycerol concentration was increased. Figure 4.3 illustrates the 1,3-PDO and butanol yield assuming that butanol was formed only by the glycerol consumed.

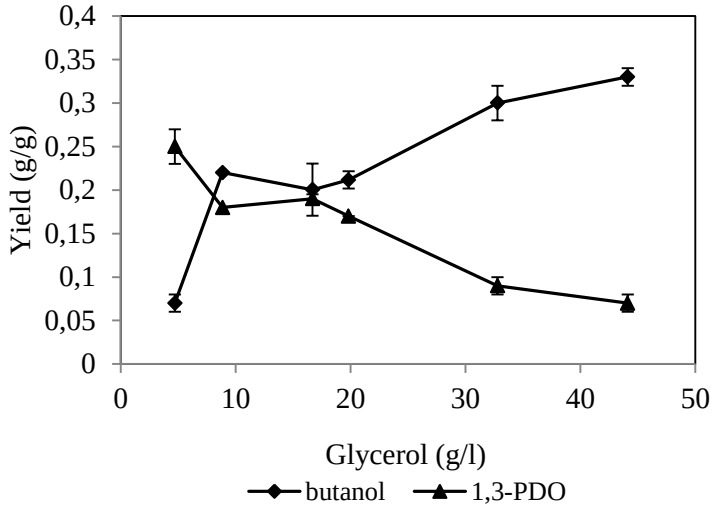

Figure 4.3. - Butanol and 1,3-PDO yield versus glycerol concentration in batch fermentation using crude glycerol and 36 mM sodium butyrate. Error bars represent standard deviation of three independent experiments

The effect of 36 mM sodium acetate on glycerol fermentation by *C. pasteurianum* DSM 525 in culture media containing 50 g l^{-1} crude glycerol is presented in Figure 4.4. Although the addition of this compound improved butanol production, its final concentration was lower than in those cultures where sodium butyrate was used. For 50 g l^{-1} crude glycerol concentration, 8.95 ± 0.27 g l^{-1} of butanol were obtained versus the 9.75 ± 0.45 g l^{-1} using sodium butyrate. Furthermore, the fermentation was longer than the control cultures and the ones in which sodium butyrate was supplemented. Similarly, glycerol consumption was not significantly affected, being 33.5 ± 1.95 g l^{-1}.

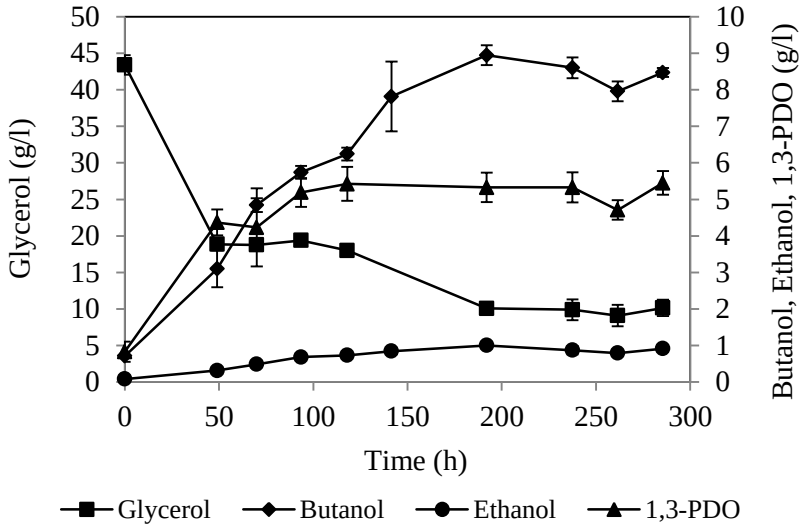

Figure 4.4. - Glycerol fermentation by *C. pasteurianum* DSM 525 using 50 g l^{-1} crude glycerol and 36 mM sodium acetate. Error bars represent standard deviation of three independent experiments

A summary of the results obtained for the experiments in which sodium butyrate was supplemented to the culture medium is presented in Table 4.4.

Table 4.4. – Crude glycerol fermentation by *C. pasteurianum* DSM 525 in culture media supplemented with 36 mM sodium butyrate. Results represent the average of three independent experiments ± S.D.

Glycerol initial ($g\ l^{-1}$)	**Glycerol consumption ($g\ l^{-1}$)**	**Butanol production ($g\ l^{-1}$)**	**Ybutanol/glycerol ($g\ g^{-1}$)**	**Ethanol production ($g\ l^{-1}$)**	**1,3-PDO production ($g\ l^{-1}$)**	**Y1,3-PDO/glycerol ($g\ g^{-1}$)**	**Acetate production ($g\ l^{-1}$)**	**Butyric acid consumption ($g\ l^{-1}$)**	**Lactate production ($g\ l^{-1}$)**
4.83 ± 0.13	4.81 ± 0.13	0.34 ± 0.04	0.07 ± 0.01	0.04 ± 0.00	1.22 ± 0.07	0.25 ± 0.02	0.22 ± 0.08	0.40 ± 0.12 (production)	0.11 ± 0.02
8.26[a]	8.26	1.86	0.22	0.13	1.52	0.18	0.21	1,03	0.09
15.71 ± 2.39	15.71 ± 2.39	3.11 ± 0.18	0.20 ± 0.03	0.25 ± 0.01	3.00 ± 0.40	0.19 ± 0.05	0.42 ± 0.13	0.36 ± 0.63	1.05 ± 0.16
19.47 ± 0.47	18.37 ± 0.50	3.91 ± 0.07	0.21 ± 0.01	0.32 ± 0.02	3.14 ± 0.09	0.17 ± 0.00	0.39 ± 0.10	0.18 ± 0,.9	1.86 ± 0.14
31.57 ± 0.58	29.01 ± 1.02	8.45 ± 0.44	0.30 ± 0.02	0.44 ± 0.06	2.59 ± 0.24	0.09 ± 0.01	0.19 ± 0.18	2.58 ± 0.20	1.80 ± 0.28
42.86 ± 0.08	27.54 ± 2.64	9.03 ± 0.45	0.33 ± 0.01	0.99 ± 0.65	1.77 ± 0.37	0.07 ± 0.01	0.03 ± 0.03	2.69 ± 0.07	1.65 ± 0.32

[a] Only one culture produced butanol due to strain degeneration

Figures 4.5 and 4.6 show a comparison of butanol and 1,3-PDO yield between cultures in which 36 mM sodium butyrate was added and controls. To determine the butanol yield, it was assumed that all butanol was formed only from the glycerol consumed.

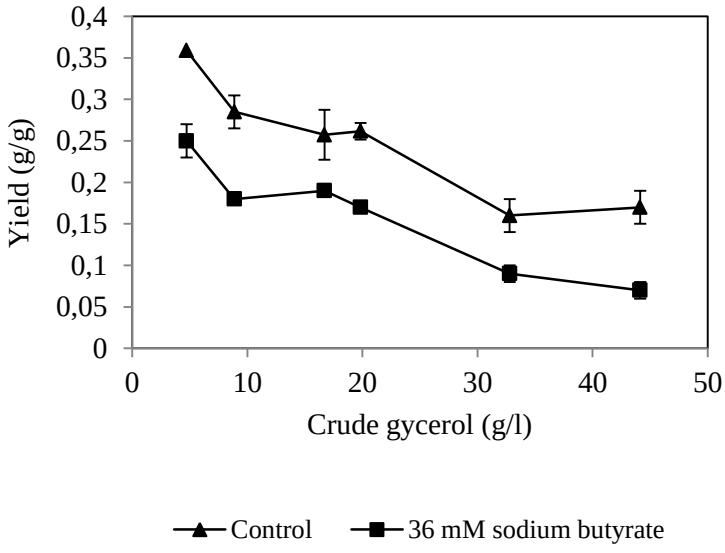

Figure 4.5. - 1,3-PDO yield on glycerol using 36 mM sodium butyrate and the control at different crude glycerol concentrations. Error bars represent standard deviation of three independent experiments

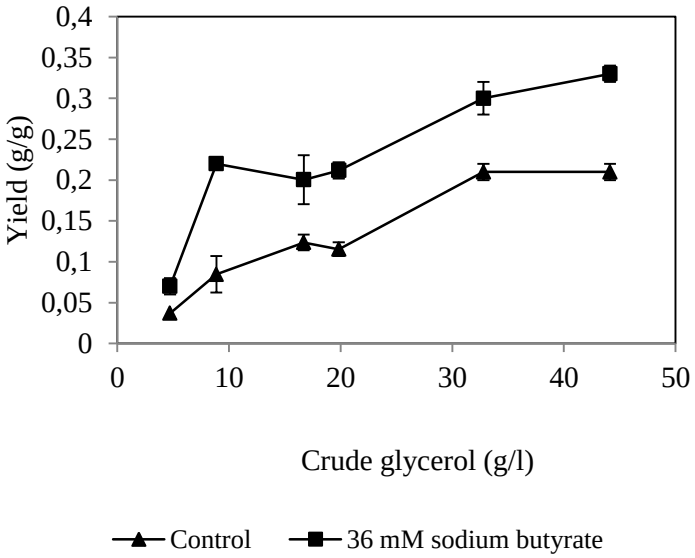

Figure 4.6. - Butanol yield on glycerol using 36 mM sodium butyrate and the control at different crude glycerol concentrations. Error bars represent standard deviation of three independent experiments

Even though more butanol could be formed from butyrate and therefore the yield of butanol reported is "apparent", an interesting fact is the lower yield of 1,3-PDO obtained. This situation shows that the supplementation of sodium butyrate in the culture medium affects the electron flow in the fermentation of glycerol by *C. pasteurianum*. The reducing equivalents formed during glycolsis are redirected to the production of butanol instead 1,3-PDO (and probably hydrogen). This situation is in accordance with the discussion presented in the section 4.1.1 and supports the hypothesis that butyrate is responsible for a higher activity of those enzymes involved in the production of butanol from butyrate.

The results obtained, which demonstrate that it is possible to obtain 9.75 ± 0.45 g l^{-1} of butanol, suggest that cells in the experiments presented in Chapter 3 (controls) did not consume more glycerol due to nutrient limitation and not due to butanol inhibition. Therefore, the evaluation of the culture medium composition was considered necessary at this point.

4.3.2 Assesment of culture medium composition

Although it was not possible to increase glycerol consumption, a simultaneous increase in $NHCl_4$ and $FeCl_2$ was found to exert a positive effect on the production of butanol. When the concentration of these compounds was increased from 3 to 5 g $l^{-1}$and from 1 to 11 mg l^{-1}, respectively, the butanol yield on glycerol increased from 0.19 ± 0.01 to 0.27 ± 0.01 g g^{-1} whereas the 1,3-PDO yield decreased from 0.15 ± 0.02 to 0.06 ± 0.01 g g^{-1}. Under this condition, the concentration of butanol and 1,3-PDO reached 9 ± 0.08 g l^{-1} and 1.78 ± 0.24 g l^{-1}, respectively. The glycerol consumption was not affected, being 29.61 ± 0.33g l^{-1} (Figure 4.7). Interestingly, these results are similar to those obtained in section 4.3.2, suggesting that iron somehow is involved in the electron flow modulation. A more detailed study about the effect of iron on the production of butanol by *C. pasteurianum* is presented in Chapter 6.

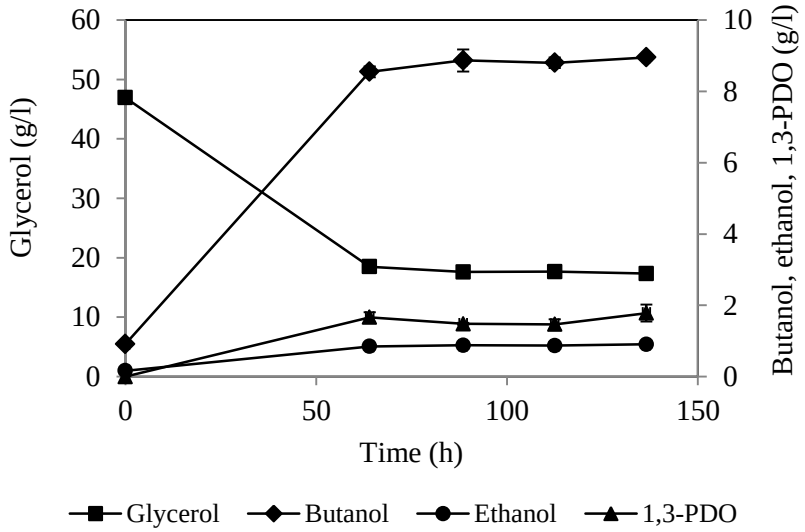

Figure 4.7. - Prodution of butanol and 1,3-PDO by *C. pasteurianum* DSM 525 using 50 g l^{-1} crude glycerol in a culture medium optimized for the production of butanol. Error bars represent standard deviation of three independent experiments

Since the butanol titer obtained using this new culture medium composition was similar to the values obtained using 36 mM sodium butyrate, and in agreement with the highest values reported in literature for batch cultures conducted in serum bottles (Malaviya et al., 2011), the next step in this work was focussed on the improvement of the tolerance of *C. pasteurianum* DSM 525 to butanol. For this purpose, random chemical mutagenesis experiments were conducted (Chapter 5).

4.4 Conclusions

The addition of either sodium butyrate or sodium acetate to the culture medium resulted in higher butanol titers in comparison with the controls. Specifically, the addition of sodium butyrate stimulates glycerol consumption and changes the product distribution towards the production of butanol. The combination of these effects resulted in higher butanol volumetric productivities.

The fact that more butanol could be produced at the expense of external added butyrate suggests that glycerol consumption in cultures without supplementation of sodium butyrate was not limited by butanol inhibition. Culture medium

optimization confirmed that hypothesis. A simultaneous increase in NH_4Cl and $FeCl_2$ were found to have a positive impact in the production of butanol.

The results gathered in this work suggest that there are other factors besides butanol inhibition and nutrient limitation that affect glycerol consumption. Also, it was demonstrated that it is possible to modulate the outcome of the fermentation towards the production of butanol by supplementing the culture medium with sodium butyrate or by adjusting the concentration of key nutrients. However, it can be concluded that the butanol tolerance of *C. pasteurianum* DSM 525 is around 9-10 g l^{-1}, which is in agreement with the information reported in the literature.

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Chapter 5

Improvement of butanol tolerance of *C. pasteurianum* DSM 525 through random mutagenesis

Butanol inhibition has been the main limiting factor for the development of an industrial process based on solventogenic clostridia. Therefore, great efforts have been done to develop butanol-resistant strains. Interesting results have been achieved. However, until recently, the lack of mutational tools and genetic information hindered the use of directed mutagenesis in *Clostridium* spp. This situation, added to the fact that butanol tolerance is a complex cellular response that involves a large number of genes, made of random mutagenesis a frequently used tool to improve the tolerance of cells to butanol. The classic random mutagenesis process involves the contact of cells with the mutagenic agent in liquid medium and subsequent selection in solid medium. Even though effective in some cases, this technique have shown to be a relatively complex process due to the number of variables involved such as mutagen concentration, time of contact, type of mutagen, among others. In this chapter, random mutagenesis in *C. pasteurianum* DSM 525 in solid medium using *N*-ethyl-*N*-nitrosourea (ENU) is studied. Experiments resulted in the isolation of cells growing in culture medium containing 12 g l^{-1} butanol, a concentration that inhibited the growth of the parent strain. Mutant cells showed 20% higher butanol production than the parent strain when grown in liquid medium. Glycerol consumption was not significantly affected. Nevertheless, a lower concentration of acids was detected at the end of the fermentation using the isolated mutant strain.

5.1 Introduction

The toxic effect that solvents, especially butanol, exert on *Clostridium* spp. limits its concentration in the fermentation broth resulting in a low titer and productivity, as well as incomplete substrate consumption. Therefore, great efforts have been made to develop butanol-tolerant solventogenic strains to overcome the above-mentioned limitations.

The solvent toxicity on clostridia has been studied by several authors (Alsaker et al, 2010; Baer et al, 1987; Borden and Papoutsakis, 2007; Costa 1981; Lepage et al. 1987; Liyanage et al., 2000; Tomas et al, 2004; Vollherbst-Schneck et al. 1984; Wang et al., 2005) and reviewed by Jones and Woods (1986), Ezeji and collaborators (2010), and Dunlop (2011). The effects of butanol on *Clostridium* spp. are complex, inhibiting several interrelated membrane processes. Costa (1981) found that longer chain alcohols are more toxic to the organisms than shorter chain alcohols. In fact, he found that a concentration of 51 g l^{-1} ethanol causes *C. acetobutylicum* to exhibit half its maximum growth rate and only 11 g l^{-1} of butanol was required to observe the same effect. Bowles and Ellefson (1985) reported that at a concentration high enough to inhibit cell growth, butanol destroyed the ability of the cell to maintain internal pH, lowered the intracellular level of ATP, and inhibited glucose uptake. Similarly, Terracciano and Kashket (1986) found that butanol dissipates the proton driving force of fermentative *C. acetobutylicum* cells by decreasing the transmembrane pH gradient. On the contrary, Wang and collaborators (2005) suggested that in *C. beijerinckii* the ΔpH across the cell membrane is dissipated in the absence of Mg^{2+} by a Na^+- or K^+-linked process, possibly by a Na^+/H^+ or a K^+/H^+ antiporter, and that the former is inhibited by butanol. Vollherbst and collaborators (1984) reported that butanol, at sub-growth-inhibitory levels, caused a ca. 20 to 30% increase in fluidity of lipid dispersions from *C. acetobutylicum*. When clostridia are exposed to butanol, the ratio of saturated to unsaturated fatty acids incorporated in the membrane lipid bilayer increases, presumably to compensate for the fluidity increase imposed by the presence of the solvent (Lepage et al., 1987).

In summary, the accumulation of solvents has several consequences. It increases the permeability and fluidity of the membrane, diminishes energy transduction and interferes with membrane protein function. The increase in membrane

permeability may result in the release of ATP, ions, phospholipids, RNA, and proteins. Direct effects include reduced ATP levels, reduced ATP synthesis, and diminished proton driving force. By affecting membrane protein function, butanol can interfere with essential cellular processes such as nutrient transport (Dunlop 2011).

The survival of microorganisms in the presence of solvent stress depends on the ability to activate a broad range of adaptation mechanisms, which function synergistically to nullify the effects of solvent toxicity on cell membranes, metabolic enzymes, and ultimately prevent loss of cell functions. (Ezeji et al, 2010). Among these mechanisms are the cell membrane composition and biophysic changes to counteract the fluidizing effects of solvents, including the short-term *cis*-to-*trans* isomerization of phospholipids, long-term changes in the ratio of unsaturated to saturated fatty acids (Baer et al. 1987; Flint and Van Dyk, 2010; Ramos et al., 1997; Weber et al., 1994) and molecular efflux pumps that help cells to detoxify the cytoplasm (Ramos et al., 2002). On the other hand, disrupted membrane processes, such as the proton driving force and associated energy production, induce changes in cellular metabolism for higher ATP production required for survival and cellular repair processes. Heat shock stress proteins (HSP) assist in protein folding and re-folding and prevent aggregation under chemical stresses. Catabolic detoxification removes toxic compounds by converting them into less harmful chemicals. Other transcriptional and translational responses may enhance viability under stress by leading to the production of osmolites like trehalose; or by upregulating specific stress systems, such as the phage-shock protein system (Nicolau et al., 2009). As an example, Borden and Papoutsakis (2007) constructed a genomic library of *C. acetobutylicum* DNA and screened it in butanol-containing media. Serial transfers to media containing progressively higher butanol concentrations enabled the enrichment of several genes, including four transcriptional regulators (CAC0977, CAC1463, CAC1869, and CAC2495). Over-expression of CAC1869 yielded an 81% improvement in butanol tolerance.

The development of solvent tolerant strains of *Clostridium* has been mainly achieved by serial enrichment using increasing butanol concentrations and random chemical mutagenesis. The reason is that solvent tolerance is a metabolic

response that involves a large number of genes (Alsaker et al, 2010; Borden and Papoutsakis, 2007; Tomas et al, 2004) and often not enough genetic information about the target microorganisms was available. Furthermore, tools for directed mutagenesis specific for *Clostridium* spp. were not available until recently (Heap et al., 2010).

Several promising results have been obtained using chemical random mutagenesis as the approach for the development of butanol tolerant strains. Hermann and collaborators (1985) isolated a mutant strain of *C. acetobutylicum* by random mutagenesis using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). The resulting mutant exhibited higher butanol resistance and about 30% more solvent production. Jain and collaborators (1993) patented an asporogenic mutant strain of *C. acetobutylicum* ATCC 4259. This strain, obtained by mutagenesis of the parent strain with aqueous EMS (ethyl methane sulfonate) solution, showed better substrate tolerance (higher than 95 g l^{-1}) and butanol production (20.2 g l^{-1}). Lemmel (1985) reported that methanesulfonic acid ethyl ester (EMS) is effective in inducing mutants of *C. acetobutylicum* resistant to ampicillin, erythromycin, and butanol (15 g/l). Malaviya and collaborators (2011) isolated a hyper butanol producing strain of *C. pasteurianum* ATCC 6103 generated by chemical mutagenesis using Nitrosoguanidine (NTG). The strain was able to produce up to 17.8 g l^{-1} butanol under experimentally optimized conditions. It is worthy to note that the same mutant strain produced 10.8 g l^{-1} of butanol under non-optimized conditions while the parent strain produced 7.6 g l^{-1}. Annous and Blaschek (1991) isolated a mutant strain of *C. beijerinkki* NCIMB 8052, called BA 101, by using NTG together with selective enrichment on the glucose analogue 2-deoxyglucose. The strain produced higher levels of amylases /glucoamylases, and also demonstrated enhanced solvent yield and total solvent production. Butanol production by *C. beijerinckii* BA101 is totally inhibited by 23 g l^{-1}, while in the parent strain this occurs at 11 g l^{-1}. The authors reported a maximum butanol production of 21 g l^{-1} in a chemically defined culture medium (MP2) containing 4.9 g l^{-1} sodium acetate and 80 g l^{-1} glucose. On the other hand, Isar and Rangaswamy (2012) reported that the optimization of physiological and nutritional parameters resulted in about 6-fold increase in butanol production by *C. Beijerinkii* NCIMB 8052 and a titre of 20 g l^{-1} of n-butanol in 72 h incubation

period. Furthermore, the strain was found to be tolerant to 25 g l^{-1} n-butanol under optimized conditions. Interesting is the fact that almost the same butanol production reported by Annous and Blaschek (1991) was achieved without any kind of genetic manipulation of *C. beijerinkii*, suggesting that operational parameters are a key factor in the production of butanol by *Clostridium* spp. that could explain the major differences in the results so far reported in literature. The authors pointed out that adaptation of the strain to increased solvent concentration in the medium was accompanied by increased expression of the chaperone GroEL, which is in agreement with the report from Tomas et al. (2003), and by a change in fatty acid profile of total lipids.

The conventional random mutagenesis process consists in exposing a microbial suspension at a given concentration, and for a given time, to a selected mutagen. The medium is then centrifuged and the microbial suspension is washed to eliminate the mutagenic agent. Next, it is cultured and then spread out on a solid nutritive medium to obtain individual colonies of surviving microorganisms. Mutants having the desired characteristics are then selected among these colonies (Hermann et al., 1988). The use of this technique, even though effective in some cases, has shown to be a relatively complex process due to the number of variables involved such as type of mutagen, time of contact, mutagen concentration, among others. Hermann and collaborators (1988) described an alternative to the classic random chemical mutagenesis process where these variables are somehow approached in a more efficient way. The process consists in spreading out a liquid culture in the growing state on solid nutritive medium containing butanol in Petri dishes. A concentration gradient of the mutagenic agent is obtained by placing a single crystal of the agent at a given point, e.g. center of the dish. Thus, through progressive diffusion it is possible to obtain the desired gradient of mutagenic agent. After incubation under appropriate conditions, the mutant strains are obtained by selecting butanol-resistant colonies. Butanol is added to the above mentioned Petri dishes at concentration which increases from dish to dish, and all of which are expected to inhibit the parent strain (Hermann et al., 1988).

The process is illustrated in Figure 5.1.

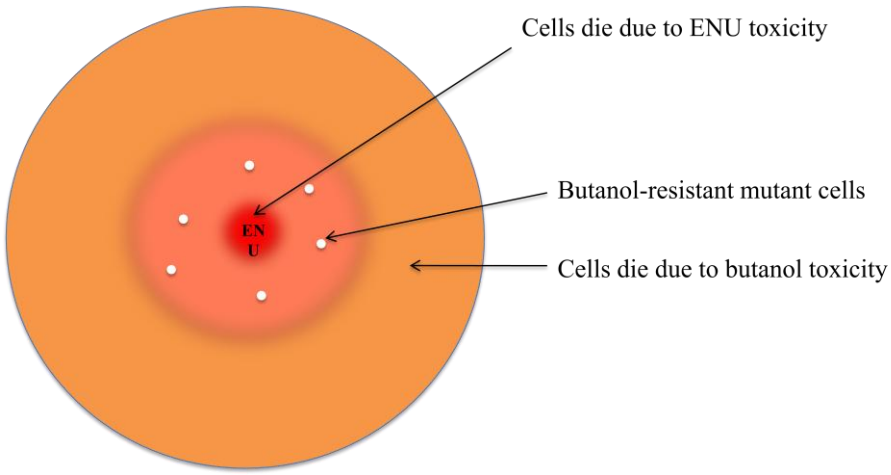

Figure 5.1. - Random mutagenesis process in solid medium

N-nitroso-*N*-ethylurea, also known as *N*-ethyl-*N*-nitrosourea or Ethylnitrosourea (ENU) is a potent monofunctional-ethylating agent that has been found to be mutagenic in a wide variety of mutagenicity test systems from viruses to mammalian germ cells (Shibuya and Morimoto, 1993). ENU possesses the dual action of ethylation and carbamoylation. The ethyl group can be transferred to nucleophilic sites of cellular constituents, and the carbonyl group can be transferred to an amino group of protein. ENU is able to produce significant levels of alkylation at oxygens, such as the O6 position of guanine and the O4 position of thymine of DNA. The molecular genetic data obtained from ENU-induced mutants on various species suggest that ENU produces mainly GC-AT transitions and, to a small extent, AT-GC, AT-CG, AT-TA, GC-CG and GC-TA base substitutions (Shibuya and Morimoto, 1993)

Even though this mutagenic agent has been used in some studies with bacteria (Javed et al., 2011; Ohta et al., 2000; Richardson et al., 1987), it has not been evaluated yet in clostridia.

In this chapter, random mutagenesis of *C. pasteurianum* DSM 525 in solid medium using ENU is studied. Potentially mutant cells are evaluated for its

capacity to produce butanol and compared with the parental strain in liquid medium.

5.2 Materials and methods

Modified Reinforced Clostridial Medium (mRCM) (Table 5.1) was prepared as described in Section 3.2.1 to ensure the absence of dissolved oxygen. The medium was dispensed in four individual 250 mL Erlenmeyer flasks and agar was added to each flask up to a final concentration of 17 g l^{-1} before autoclaving them for 20 min. The culture media were let to cool down and butanol was aseptically added before it became solid. Butanol concentrations used were 0, 12, 15 and 20 g l^{-1}.

Four disposable Petri dishes were prepared for each butanol concentration. The plates were placed inside a vinyl anaerobic chamber (Coy Laboratory Products) at least three hours before the mutagenesis experiment was conducted to ensure the absence of oxygen.

Table 5.1. - Modified Reinforced Clostridial Medium (mRCM)

Compound	**Concentration (g l^{-1})**
Beef extract	10
Peptone	10
Sodium chloride	5
Glucose	20
Yeast extract	3
Sodium acetate	3
Soluble starch	1
L-cysteine $HCl.H_2O$	0.56

Random mutagenesis in *C. pasteurianum* DSM 525 was conducted as described by Hermann and collaborators (1985; 1988) with minor modifications. A crystal of ENU was placed at the center of each plate containing mRCM supplemented with butanol. Two plates without mutagen were used as control for each butanol concentration tested. After 15 min, 200 µl of an exponential-phase culture of *C. pasteurianum* DSM 525 grown in mRCM were spread out on each plate.

The plates were then incubated at 37°C inside an anaerobic box until growth was observed (typically between 48 and 72 h). Colonies growing at butanol

concentrations that did not allow growth of the parent strain (controls) were selected and purified by transferring them to plates containing the same medium composition and butanol concentration but without the mutagenic agent. Colonies thus obtained were transferred to 20 ml liquid mRCM, and after 24 hours a volume of 6 ml cell suspension was used to inoculate 60 ml of the culture medium presented in table 5.2 in order to test their ability to produce butanol and to compare them with the parent strain. Experiments were conducted in triplicate. Samples were taken at regular time intervals and products were analyzed as described in section 3.2.2.

Table 5.2. - Culture medium used to evaluate potential mutant strains obtained through random mutagenesis

Compound	**Concentration**
Pure glycerol	50 g l^{-1}
KH_2PO_4	0.5 g l^{-1}
K_2HPO_4	0.5 g l^{-1}
$MgSO_4.7H_2O$	0.2 g l^{-1}
$FeSO_4.7H_2O$	11 mg l^{-1}
$CaCl_2.2H_2O$	0.02 g l^{-1}
$CaCO_3$	3 g l^{-1}
Cysteine-$HCl.H_2O$	0.5 g l^{-1}
NH_4Cl	5 g l^{-1}
Yeast extract	1 g l^{-1}
Resazurin	0.5 mg l^{-1}
Trace solution 6 (Table 3.2)	1 ml l^{-1}
Trace solution 7 (Table 3.3)	1 ml l^{-1}

5.3 Results and discussion

After 72 hours, two colonies were obtained in plates containing 12 g l^{-1} butanol and the mutagen. No growth was observed in the control plates, which is in agreement with the results previously obtained in this thesis, as well as in the information reported in literature. These colonies were purified and a total of nine colonies were transferred to liquid medium for testing their ability to produce solvents, as well as to compare them with the parent strain as explained in Section 5.2. The results obtained are presented in Table 5.3.

Table 5.3. – Glycerol fermentation by mutant strains obtained through random mutagenesis and the parent strain. Results represent the average of three independent experiments ± S.D.

Substrate/product (g l^{-1})	M 1	M2	M3	M4	M5	M6	M7	M8	M9	MC
Glycerol initial	45.26 ± 4.03	46.10 ± 4.07	45.80 ± 3.69	45.56 ± 5.87	45.91± 5.34	43.20 ± 3.95	46.84 ± 1.98	45.09 ± 2.68	46.52 ± 0.93	46.38 ± 3.66
Glycerol final	10.67 ± 1.92	9.97 ± 1.69	10.68 ± 1.68	9.08 ± 4.98	10.02 ± 3.74	14.62 ± 2.92	10.30 ± 2.66	10.40 ± 1.65	10.44 ± 1.50	11.78 ± 1.13
Glycerol consumption	34.59 ± 2.12	36.13 ± 2.38	35.12 ± 2.01	36.48 ± 0.89	35.88 ± 1.60	28.58 ± 1.03	36.54 ± 0.68	34.68 ± 1.03	36.09 ± 0.57	34.60 ± 2.53
Butanol initial	0.39 ± 0.01	0.40 ± 0.00	0.39 ± 0.01	0.41 ± 0.05	0.38 ± 0.01	0.32 ± 0.00	0.40 ± 0.01	0.39 ± 0.01	0.38 ± 0.03	0.31 ± 0.02
Butanol final	8.07 ± 0.38	8.72 ± 0.07	8.05 ± 0.23	8.53 ± 0.03	8.37 ± 0.15	7.13 ± 0.07	8.08 ± 0.19	7.98 ± 0.16	7.93 ± 0.40	7.13 ± 0.13
Butanol yield	0.22 ± 0.00	0.23 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.24 ± 0.01	0.21 ± 0.01	0.22 ± 0.00	0.21 ± 0.01	0.20 ± 0.01
1,3-PDO initial	0	0	0	0.14 ± 0.18	0.03 ± 0.04	0	0	0	0	0
1,3-PDO final	7.52 ± 0.16	7.25 ± 0.32	7.38 ± 0.62	7.88 ± 0.33	8.25 ± 0.44	4.91 ± 0.72	8.53 ± 0,06	7.47 ± 0.81	7.65 ± 0.74	6.79 ± 0.23
1,3-PDO yield	0.22 ± 0.01	0.20 ± 0.00	0.21 ± 0.01	0.21 ± 0.01	0.23 ± 0.00	0.17 ± 0.02	0.23 ± 0.01	0.22 ± 0.03	0.21 ± 0.02	0.20 ± 0.01
Acetate initial	0	0	0	0	0	0	0	0	0	0
Acetate final	0.72 ± 0.02	0.60 ± 0.00	0.73 ± 0.08	0.77 ± 0.00	0.85 ± 0.04	0.69 ± 0.02	0.87 ± 0.03	0.83 ± 0.01	0.80 ± 0.04	0.87 ± 0.07
Butyrate initial	0	0	0	0	0	0	0	0	0	0
Butyrate final	0.33 ± 0.04	0.14 ± 0.01	0.64 ± 0.04	0.16 ± 0.23	0.59 ± 0.16	1.29 ± 0.45	0.86 ± 0.10	0.77 ± 0.13	0.42 ± 0.04	0.90 ± 0.00
Lactate initial	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00
Lactate final	1.48 ± 0.04	1.28 ± 0.08	1.22 ± 0.09	1.55 ± 0.03	1.61 ± 0.06	1.70 ± 0.63	1.78 ± 0.01	1.59 ± 0.12	1.30 ± 0.24	1.64 ± 0.09

Nomenclature:
M1-M9: Cultures started from nine colonies isolated in solid medium containing 12 g l^{-1} butanol (mutants); MC: Control - parent strain used in the mutagenesis experiment

As shown in table 5.3, the isolated strain called M2 produced the highest amount of butanol, corresponding to a final titer of 8.72 ± 0.07 g l^{-1}. Even though, this is not the highest butanol titer obtained in this PhD work, it represents a 20% increase and 15% in the yield in comparison with the control (MC) under the same culture conditions. The percentual increase in butanol production is similar to the results reported by Hermann and collaborators (1985) using the same mutagenic process with *C. acetobutylicum* and NTG as the mutagenic agent (around 19 %). It is worth to note that an increase in solvent tolerance not always leads to a higher butanol production (Dunlop, 2011). However, this particular random mutagénesis technique seems to be suitable to obtain butanol overproducing strains as it was pointed out by Hermann et al. (1988).

In terms of glycerol consumption, there was not an appreciable difference between the strain M2 and the control (36.13 ± 2.38 versus 34.60 ± 2.53 in the control). Furthermore, 1,3-PDO yields were the same for both strains (0.2 g g^{-1}), while a lower concentration of acids, particularly butyrate was observed at the end of the fermentation using the strain M2.

The lower final concentration of acids obtained with the mutant strain could indicate that either a higher amount of these acids (acetic and butyric) was reassimilated during the fermentation, thus leading to the higher butanol titer observed; or simply a lower producion of butyric acid and more butanol produced via pyruvate- butyryl CoA – butyraldehyde –butanol (Figure 2.3).

Interestingly, the results herein obtained are similar to the ones reported by Formanek et al. (1997) with the mutant strain *C. beijerinckii* BA101. The authors found that even though the initial levels of acids produced by BA101 were comparable to those observed for the parent strain (*C. beijerinckii* NCIMB 8052), these values decreased dramatically over the course of the fermentation, thus suggesting that the strain BA101 may be a superrecycler of butyric and acetic acids, thereby detoxifying the cellular environment and contributing additional carbon to the production of neutral solvents.

On the other hand, the maximum butanol concentration observed in the control (7.13 ± 0.13) was lower than the values obtained in previous experiments (Chapter 4). This situation is attributed to differences in culture conditions. Malaviya and collaborators (2012) reported that several factors such as inoculum

age, initial pH and pH control are critical variables that directly influence the production of butanol from glycerol by *C. pasteurianum* DSM 525. Biebl (2001) reported variability in product formation under seemingly equal or slightly varied conditions using *C. pasteurianum* DSM 525. The author suggested that the regulation of the numerous fermentation pathways occurring in this organism is not very strict.

In order to study the butanol tolerance of the strain M2, cells were challenged with different concentrations of butanol (0, 5, 10, and 15 g l^{-1}). Basically, an exponential-phase culture of the strain was used to inoculate liquid culture media (Table 5.2 - medium containing 80 g l^{-1} glycerol) containing the desired butanol concentrations. The strain M2 was able to grow in culture medium containing up to 10 g l^{-1} butanol, reaching a final butanol concentration of 12.06 ± 0.15 g l^{-1}. This experiment confirms the results obtained in solid medium. Nevertheless, it is important to notice that in culture medium without added butanol, the final concentration of butanol was 8.77 ± 0.23 g l^{-1}, therefore other variables must be affecting the cellular metabolism as it was pointed out previously.

Based on the results obtained and the ones reported by Malaviya and collaborators (2011), the effect of iron and inoculum age on glycerol fermentation using the mutant strain M2 will be further explored (Chapter 6).

5.4 Conclusions

The random mutagenesis technique used in this experiment proved to be an efficient tool as alternative to the classical random mutagenesis in liquid medium. The use of ENU as the mutagen agent, which has not been reported before for *Clostridium* spp., allowed isolating cells growing at 12 g l^{-1} butanol in solid medium, a concentration that is inhibitory for the parent strain. Mutant cells showed 20% higher butanol production than the parent strain in liquid medium. When challenged with butanol, the isolated strain was able to grow up to a concentration of 10 g l^{-1} of butanol, resulting in a final titter of 12.06 ± 0.15 g l^{-1} which is higher than the values reported in literature for *C. pasteurianum* DSM 525 grown on glycerol in serum bottles. The data obtained suggest that other factors different from nutrient limitation and inhibition by butanol are affecting

butanol production by *C. pasteurianum* which can justify the major differences that are observed and reported in the literature even when apparently the same culture conditions are used.

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Chapter 6

Effect of iron concentration and inoculum age on the production of butanol from glycerol by *C. pasteurianum*

The concentration of iron and the inoculum age have been recognized as important variables in the production of butanol by *C. pasteurianum*. Iron is a key element present in at least four important proteins involved in the production of hydrogen and butanol. On the other hand, the inoculum age is related with the cell concentration and its physiological state. In this chapter, both variables are studied and results confirmed the information reported in literature and Chapter 4. Iron limitation led to the inhibition of butanol production. The supplementation of the culture medium with only 3 mg l^{-1} of $FeSO_4.7H_2O$ yielded a 140 % increase in butanol production. Nevertheless, there were no appreciable differences in butanol production using higher iron concentration ranging from 10 to 100 mg l^{-1}. The inoculum age showed an effect on both glycerol consumption and butanol production. The optimal inoculum age was found to be 12 hours leading to 45.62 ± 3.81 g l^{-1} glycerol consumption and 12.4 ± 0.26 g l^{-1} butanol titter using an optimal iron concentration. These values are higher than the previous results obtained in this PhD work under non-optimized conditions and than the results reported in literature on butanol production from glycerol by *C. pasteurianum* in serum bottles.

6.1 Introduction

6.1.1 Effect of iron

The concentration of iron in the culture medium has been recognized as an important factor in solvent production by clostridia. This element is part of at least four important enzymes, namely piruvate-ferredoxin oxidoreductase (Gheshlaghi et al, 2009), ferredoxin (Graves et al, 1985), hydrogenases I and II (Adams et al., 1989) and iron-containing alcohol dehydrogenase family proteins (*C. pasteurianum* DSM 525 genome annotation).

During the course of glucose fermentation by solventogenic *Clostridium* spp., the electron flow is first directed to hydrogen production and the carbon flow to acid biosynthesis. The pyruvate resulting from glycolysis is cleaved by pyruvate-ferredoxin oxidoreductase (PFOR) in presence of coenzyme-A to yield carbon dioxide and acetyl-CoA with concomitant conversion of oxidized ferredoxin to its reduced form (Menon and Ragsdale, 1997; Gheshlaghi et al, 2009). PFOR contains an iron–sulfur-chromophor in the redox center that shuttles the electron flow from pyruvate to ferredoxin (Gheshlaghi et al, 2009). *Clostridium* ferredoxin (Fd) contains two [4Fe:4S] clusters and serves as a two-electron carrier for a host of metabolic redox reactions such as hydrogen production. *Clostridium* grown in iron-sufficient media produce yields of Fd up to 2% of the total cell protein (Graves and Rabinowitz, 1986). Hydrogenase from *C. pasteurianum* W5, which oxidizes reduced ferredoxin for the production molecular hydrogen, was found to have 4-5 iron and 4-5 labile sulphur atoms per molecule of 60,000 molecular weight (Erbes et al, 1975). On the other hand, in the solventogenic phase both electron and carbon flows are directed to solvent production via ferredoxin $NAD(P)^+$ reductase and aldehyde and alcohol dehydrogenases (Junelles et al, 1988). Butanol dehydrogenase catalyzes the final step in butanol synthesis, the reduction of butyraldehyde to butanol at the expense of a reduced NAD(P). The activity of these enzymes is a key factor in the regulation of carbon and electron flow towards the production of hydrogen or butanol during the fermentation.

Preliminary experiments in this PhD work (Chapter 4) showed that an increase in iron concentration in the culture medium led to a higher butanol production. In

this chapter, the effect of iron concentration in the culture medium on the fermentation of glycerol by *C. pasteurianum* is evaluated.

6.1.2 Inoculum age

The inoculum age has been also considered as an important variable in microbial fermentation that directly influences cell growth, productivity and reproducibility of fermentations. The reason for this is that microorganisms, depending on their physiological state, react in a different way to pH fluctuations and stress conditions that are involved in their transference to fresh culture medium (Hornbaek et al., 2004).

A special feature of bacteria belonging to the Clostridium genus (among others) is its life cycle, which includes a vegetative stage, endospore formation and spore release (Figure 6.1). The production of butanol by *Clostridium* spp. has shown to be directly related to the physiological state of the cells. A classic example is the biphasic fermentation of sugars by *C. acetobutylicum*. In this microorganism the initiation of solvent production and sporulation coincide and are both regulated by the protein SpoOA (Harris et al., 2002; Ravagnani et al., 2000).

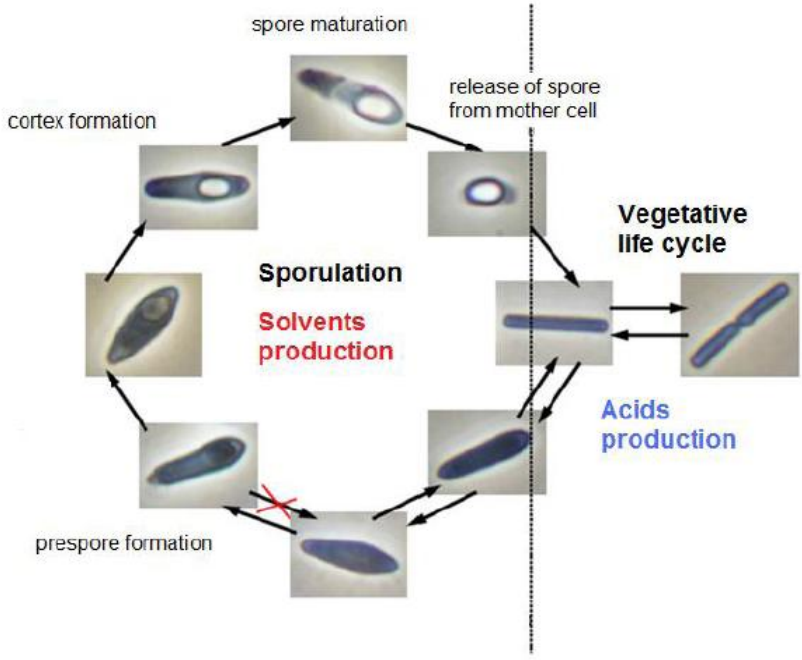

Figure 6.1. - Life cycle of *Clostridium* spp. Source: Patakova et al. (2011)

A common practice in most fermentation processes is to use exponentially growing cells as inoculum. In this way, the lag phase can be shortened or even eliminated, thus increasing the volumetric productivity of the fermentation. However, the situation is somehow different for *Clostridium* spp. Due to the possibility of strain degeneration during repeated subculturing when cells are kept at vegetative stage, the standard practice has been to maintain solvent-producing clostridia as spores, which can be heat activated and germinated when an inoculum is required (Jones and Woods, 1986). The fact that the transfer of exponentially growing *Clostridium* cells is unsual led to some negligence, with few exceptions, in the evaluation of the effect of the inoculum age on the production of butanol.

Gutierrez and Maddox (1987a) found a significant improvement in solvent production when *C. acetobutylicum* culture transfers during the inoculum development procedure were made at the time of maximum cell motility. In another work, the same authors (1987b) studied the role of chemotaxis in solvent production by *C. acetobutylicum*. They found that virtually 100% of the cells remained motile until about 13 hours, and postulated that strongly motile cells are are more solventogenic than weakly motile cells because they are attracted to fermentable sugars and undissociated acids while being repelled by the solvents.

Malaviya and collaborators (2011) showed that the inoculum age, ultimately related with cell concentration and the physiological state of the cell population, is an important variable that somehow influences the final butanol titer attainable by *C. pasteurianum*. In this Chapter, the effect of this parameter is also studied.

6.2 Materials and methods

C. pasteurianum (strain M2 obtained in Chapter 5) was cultured in 160 ml serum bottles containing 60 ml of the culture medium presented in Table 5.2 but using 90 g l^{-1} pure glycerol. Also, a modified trace elements solution 6 without $FeCl_2$ was used to study the effect of iron concentration. The culture medium was prepared as described in Section 3.2.1. Six ml of an exponential-phase culture (or as indicated to study the effect of inoculum age) were used as inoculum.

To study the effect of iron, the culture medium was supplemented with different concentrations of $FeSO_4.7H_2O$. The concentrations used were 0, 3, 10, 50, and 100 mg l^{-1}.

To study the effect of the inoculum age, cells were transferred to fresh medium at 12, 16, 20, 24, and 36 hours after inoculation.

Experiments were conducted in triplicate. Samples were taken at regular intervals and products were measured as it is described in Section 3.2.2.

6.3 Results and discussion

6.3.1 Effect of iron

Results about the effect of iron concentration in the culture medium on glycerol fermentation by *C. pasteurianum* M2 are presented in table 6.1

Table 6.1. - Effect of iron concentration on glycerol fermentation by *C. pasteurianum* M2. Results represent the average of three independent experiments ± S.D.

	$FeSO_4.7H_2O$ (mg l^{-1})				
	0	**3**	**10**	**50**	**100**
Glycerol initial (g l^{-1})	91.89 ± 1.04	93.61 ± 1.33	94.41 ± 2.43	89.90 ± 2.67	89.21 ± 3.97
Glycerol final (g l^{-1})	63.81 ± 1.5	59.43 ± 0.57	58.88 ± 1.87	57.06 ± 0.91	55.78 ± 5.00
Glycerol consumption (g l^{-1})	28.09 ± 2.54	34.18 ± 0.76	35.52 ± 0.55	32.83 ± 3.58	33.43 ± 8.98
Butanol initial (g l^{-1})	0.06 ± 0.00	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.00
Butanol final (g l^{-1})	3.17 ± 0.20	7.64 ± 0.37	8.34 ± 0.21	8.51 ± 0.16	8.27 ± 1.64
Butanol yield (g g^{-1})	0.11 ± 0.00	0.22 ± 0.02	0.23 ± 0.01	0.26 ± 0.02	0.25 ± 0.02
1,3-PDO initial (g l^{-1})	0	0	0	0	0
1,3-PDO final (g l^{-1})	5.21 ± 0.45	5.04 ±0.36	4.86 ± 0.25	4.67 ± 0.29	4.77 ± 1.05
1,3-PDO yield (g g^{-1})	0.19 ± 0.00	0.15 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.01
Ethanol initial (g l^{-1})	0	0	0	0	0
Ethanol final (g l^{-1})	0.28 ± 0.02	0.80 ± 0.02	0.67 ±0.13	0.62 ± 0.03	0.65 ± 0.05
Acetic acid initial g l^{-1})	0	0	0	0	0
Acetic acid final (g l^{-1})	0.50 ± 0.07	0.26 ± 0.02	0.29 ± 0.02	0.24 ± 0.00	0.22 ± 0.02
Butyric acid initial (g l^{-1})	0	0	0	0	0
Butyric acid final (g l^{-1})	0.80 ± 0.18	0.10 ± 0.14	0.08 ± 0.11	0.08 ± 0.11	0.07 ± 0.08
Lactic acid initial (g l^{-1})	0	0	0	0	0
Lactic acid final (g l^{-1})	2.25 ± 0.22	1.69 ± 0.39	1.51 ± 0.05	1.42 ± 0.05	1.30 ± 0.12
pH initial	6.64 ± 0.02	6.65 ± 0.00	6.65 ± 0.00	6.63 ± 0.00	6.62 ± 0.01
pH final	4.68 ± 0.01	5.04 ± 0.11	5.07 ± 0.06	5.06 ± 0.04	5.02 ± 0.08

As it is shown in Table 6.1, the iron concentration influences both butanol production and glycerol consumption. Iron limitation led to a lower glycerol consumption and solvent production. The supplementation of only 3 mg l^{-1} $FeSO_4.7H_2O$ yielded 140 % increase in butanol production. Nevertheless, further increase in $FeSO_4.7H_2O$ did not result in appreciable differences.

Besides the effect observed on glycerol consumption and butanol production, two other differences can be observed in Table 6.1. When $FeSO_4.7H_20$ was not supplemented to the culture medium (iron limitation), a lower pH and higher concentration of acids was obtained at the end of the fermentation. The results suggest that the lower pH reached at the end of the fermentation under this condition was due to a lower re-assimilation of acids (acetic and butyric), probably as a consequence of a reduced activity of alcohol dehydrogenases. However, the higher lactate concentration observed indicates that the conversion of piruvate to acetyl CoA is partially blocked what eventually can contribute to the lower pH observed by limiting the production of hydrogen.

Even though lactic acid often is not a major fermentation product in *Clostridium* spp. (Andreesen et al., 1969; Gheshlaghi et al, 2009), it has been reported that its production can be important under stressful conditions such as iron limitation (Bahl et al., 1986; Gheshlaghi et al, 2009). Lactate is produced due to a parcial blockage in the conversion of pyruvate to acetyl-CoA, reaction catalized by pyruvate-ferredoxin oxidoreductase. The blockage of pyruvate increases the intracellular concentration of fructose -1,6- diphosphate (F1,6DP), which in turn activates lactate dehydrogenase (Dürre, 2005; Gheshlaghi et al, 2009), a nonreversible enzyme that reduces pyruvate to lactate and is activated by F1,6DP (Contag et al., 1990; Gheshlaghi et al, 2009).

The results herein obtained are in agreement with the ones reported by Dabrock and collaborators (1992). The authors studied the effect of iron limitation in the fermentation of glycerol by *C. pasteurianum* DSM 525. They found that iron limitation somehow inhibits the formation of butanol and ethanol favoring the production of lactate, and suggested the possibility that alcohol dehydrogenases involved in the process are iron dependent.

On the other hand, the results obtained are opposite to the ones reported by Junelles et al. (1988) using *C. acetobutylicum*. The author found that when this

microorganism was grown under iron limitation (0.2 mg l^{-1}) the conversion yield of butanol could be increased from 20% to 30% working at pH 4.8. Peguin and Soucaille (1995) also reported a higher butanol production and yield on glucose using *C. acetobutylicum* under iron limitation. However, the culture medium composition and pH could influence the results. Junelles and collaborators (1988) and Peguin and Soucaille (1995) worked in fermentors with pH control using glucose as the carbon source, while the results presented by Dabrock et al. (1992) and in this thesis were obtained from experiments in serum bottles without pH control using glycerol as the carbon source. The culture medium used in this thesis contained 1 g l^{-1} yeast extract. However, if we consider an average Fe content of 0.15 mg g^{-1} dry wt. yeast extract (Grant and Pramer, 1962), it is possible to estimate that the real iron concentration of the culture medium herein used without $FeSO_4.7H_2O$ supplementation is 0.15 mg l^{-1}, which can be considered in the limitation range based on the considerations of Junelles and collaborators (1988) and the glycerol concentration used.

6.3.2 Effect of inoculum age

The effect of the inoculum age on the production of butanol from glycerol by *C. pasteurianum* M2 is presented in Table 6.2.

Table 6.2. - Effect of inoculum age in the production of butanol from glycerol by *C. pasteurianum* M2. Results represent the average of three independent experiments ± S.D.

	Inoculum age (h)				
	12	**16**	**20**	**24**	**36**
Glycerol initial (g l^{-1})	86.68 ± 1.55	86.94 ± 0.51	88.20 ± 3.77	83.66 ± 0.57	83.61 ± 0.51
Glycerol final (g l^{-1})	41.06 ± 5.36	45.74 ± 1.99	49.21 ± 6.01	45.47 ± 1.34	48.20 ± 1.81
Glycerol consumption (g l^{-1})	45.62 ± 3.81	41.20 ± 1.49	38.99 ± 2.24	38.18 ± 1.92	35.40 ± 2.32
Butanol initial (g l^{-1})	0.04 ± 0.05	0.09 ± 0.01	0.08 ± 0.02	0.09 ± 0.03	0.08 ± 0.01
Butanol final (g l^{-1})	12.40 ± 0.26	11.68 ± 0.12	10.53 ± 2.21	10.76 ± 0.28	9.94 ± 0.84
Butanol yield (g g^{-1})	0.26 ± 0.02	0.28 ± 0.01	0.27 ± 0.04	0.28 ± 0.01	0.28 ± 0.01

The inoculum age was found to have a significant impact on glycerol consumption and butanol production by *C. pasteurianum*. Twelve hours was found to be the optimal time (inoculum age). Using this condition, an improvement in glycerol consumption and butanol production was achieved, resulting in 45.62 ± 3.81 and 12.40 ± 0.26 g l^{-1}, respectively. The concentration of 1,3-PDO reached 7.45 ± 0.86 g l^{-1}.

Malaviya and collaborators (2011) also studied the effect of inoculum age, besides initial cell density and initial pH, on the production of butanol by a hyper-butanol producing mutant strain of *C. pasteurianum* obtained through random chemical mutagenesis. All three variables showed to have a great impact on the production of butanol. The authors reported an optimal inoculum age of 18 hours. Nevertheless, comparing their results on butanol production obtained at 18 hours and 12 hours the differences were not very significant (6.6 ± 0.16 *versus* 5.8 ± 0.08 gl^{-1}). On the contrary, the authors reported that a 24 hours inoculum age led to less than a half the concentration of butanol (3 ± 0.25 g l^{-1}). Even though, the results obtained in the current work clearly show that butanol production decreases with the inoculum age, the difference between the optimal (12 h) and 24 hours was not as pronounced as reported by Malaviya and collaborators (2011).

Interestingly, the results obtained are in agreement with the experiments reported by Gutierrez and Maddox (1987a) and mentioned in Section 6.1.2. The optimal inoculum age herein found (12 hours) is within the range that the authours reported as the time in which *C. acetobutylicum* cells remain motile. However, the relation between cell motility, solvent capacity and chemotaxis should be further studied.

6.4 Conclusions

The concentration of iron and the inoculum age were identified as key factors in the fermentation of glycerol by *C. pasteurianum*. Results suggest that the lower pH reached at the end of the fermentation under iron limitation is a consequence of the lower re-assimilation of acids (butyric and acetic), probably due to a low activity of alcohol dehydrogenases. On the other hand, the higher lactic acid

concentration observed under iron limitation can be explained by a low activity of pyruvate-ferredoxin oxidoreductase.

An optimal inoculum age of 12 hours was found, which led to an increase in glycerol consumption and butanol production. The values obtained represent an improvement as compared to previous experiments. Particularly, the butanol titer obtained is higher than the values reported in literature on the production of butanol by *C. pasteurianum* in serum bottles and are also in agreement with the butanol tolerance of the strain (Chapter 5).

6.5 References

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Chapter 7

Effect of pH and N_2 sparging on glycerol fermentation by *C. pasteurianum*

The pH and N_2 sparging have been recognized as important variables in the production of solvents by *Clostridium* spp. In particular, it has been shown that fermentations in pH–controlled reactors have resulted in a higher glycerol consumption and butanol titer using *Clostridium pasteurianum*, although the reason behind it is still not clear. On the other hand, N_2 sparing has shown to be a useful way to relieve hydrogenase inhibition by H_2 dissolved concentration thus affecting the product distribution in the ABE fermentation. In the experiments presented in this Chapter, the effect of N_2 sparging on the fermentation of glycerol by *C. pasteurianum* was evaluated at pH 6.0 and the effect of pH was also evaluated in a fermentation conducted at pH 5.0. The experiment conducted at pH 6.0 where N_2 sparging was not used, resulted in lower glycerol consumption but a higher butanol yield in comparison with the fermentation at the same pH, using 0.5 vvm N_2. In the experiment conducted at pH 5.0, the glycerol consumption and biomass concentration were found to be much lower and a longer fermentation time was also observed as compared with the pH 6.0 experiment. Nevertheless, this condition led to the higher butanol yield (0.22 g g^{-1}). Even though it was possible to achieve high glycerol consumption (75 g l^{-1}) in the fermentation controlled at pH 6.0 in which N_2 sparging was used, the butanol titer was always below 9.5 g l^{-1}. Under this condition, the 1,3-PDO concentration reached almost 20 g l^{-1}. The maximum butanol volumetric productivity obtained was 0.29 g $l^{-1}h^{-1}$, which is comparable to the values reported in literature for batch cultures using *Clostridium* spp. Results suggest that the dissolved H_2 concentration is a critical parameter in the fermentation of glycerol by *C. pasteurianum* that indeed controls the product distribution and is affected by N_2 sparging. Furthermore, the importance of pH on the fermentation was confirmed. Overall, the production of butanol from glycerol by *C. pasteurianum* shows a different behaviour from the one observed in the fermentation of sugars by other solventogenic clostridia.

7.1 Introduction

Even though the use of serum bottles for batch culture are advantageous in terms of handling and the number of experiments that can be conducted in parallel, scale-up of fermentation processes for the production of bulk chemicals is required in order to make experiments closer to an industrial process, and also to accurately evaluate how production parameters can be affected. On the other hand, the use of laboratory-scale bioreactors allows a better control of some key fermentation parameters such as pressure, pH and agitation.

Laboratory-scale bioreactors offer the possibility of conducting fermentations at atmospheric pressure by using an inert gas to maintain the anaerobic conditions required by *Clostridium* spp. On the contrary, serum bottles are commonly overpressured and periodic release of gas is required when cells start to grow. This situation leads to an increase in the H_2 and CO_2 partial pressure, which in turn could affect the outcome of the fermentation by inhibiting key enzymes, as it has been reported before (Doremus et al., 1985).

The pH of the fermentation has been reported as an important variable in butanol production by *Clostridium* spp. However, the optimal pH range for solvent production varies significantly among species and strains. Furthermore, it has been shown that a low pH by itself does not trigger the onset of solventogenesis (Jones and Woods, 1986). Interestingly, in *C. pasteurianum* the fermentation of glycerol in pH-controlled reactors has yielded much higher butanol titers than in serum bottles. As an example, Malaviya and collaborators (2011) studied the effect of the initial pH on butanol production in anaerobic bottles using a hyper-butanol producing mutant strain of *C. pasteurianum* DSM 525 obtained by random chemical mutagenesis. The authors found that the optimal initial pH was 5.5, resulting in a final butanol titer of 7.8 ± 0.4 g l^{-1} using a culture medium containing 80 g l^{-1} pure glycerol. The butanol titer decreased considerably at pH 4.5 (0.5 ± 0.3 g l^{-1}) and 7.0 (4.1 ± 0.4 g l^{-1}), which is the pH value recommended for optimal growth of the wild type strain. The maximum butanol titer that the authors achieved in anaerobic bottles using the mentioned mutant strain, and after the optimization of the initial cell density, was 9.4 ± 0.02 g l^{-1}. However, when cells were grown in a pH-controlled reactor (pH ≥ 4.8) the butanol titer increased to 15.5 g l^{-1}. Under optimized conditions the authors reported a final butanol

concentration of 17.8 g l^{-1}, the higher butanol titer ever reported for *C. pasteurianum*. In another study, Biebl (2001) reported 17 g l^{-1} of butanol using *C. pasteurianum* DSM 525 without any genetic manipulation in a medium containing 80 g l^{-1} pure glycerol. A common feature in both works is that pH was controlled during the fermentation. On the other hand, all reports on butanol production from glycerol using *C. pasteurianum* DSM 525 in serum bottles, where pH cannot be accurately controlled, have showed butanol titers lower than 10 g l^{-1}.

Agitation is another important variable directly related with the mass transfer phenomenon that can be controlled in a lab-scale bioreactor. By the use of an impeller (typically Rushton turbine), it is possible to apply a desired degree of mixing. Even though agitation is more important in aerobic fermentations, where the supply of oxygen to microorganisms is critical, in anaerobic fermentations it allows a better mixing thus eliminating concentration gradients that could be present in serum bottles. Moreover, it has been shown that agitation affects the level of dissolved hydrogen gas in the culture medium, which in turn influences solvent production in the acetone-butanol fermentation (Doremus et al., 1985; Yerushalmi and Volesky, 1985).

In this Chapter, the effect of pH and N_2 sparging on the fermentation of glycerol by *C. pasteurianum* are studied.

7.2 Materials and methods

The experiments were conducted in a 1.5 L stirred tank reactor (Autoclavable benchtop fermentor Type R'ALF, Bioengineering AG, Wald, Switzerland), equipped with two Rushton flat blade turbines, pH and temperature control. The working volume used was 1.2 L. The composition of the culture medium used is presented in Table 7.1.

Table 7.1. - Culture medium used to study the effect of pH and N_2 sparging on the fermentation of glycerol by *C. pasteuriaum*

Compound	Concentration
Pure glycerol	90 g l^{-1}
KH_2PO_4	0.5 g l^{-1}
K_2HPO_4	0.5 g l^{-1}
$MgSO_4.7H_2O$	0.2 g l^{-1}
$FeSO_4.7H_2O$	50 mg l^{-1}
Cysteine-HCl.H_2O	0.5 g l^{-1}
NH_4Cl	5 g l^{-1}
Yeast extract	1 g l^{-1}
Resazurin	0.5 mg l^{-1}
Trace elements solution 6 (Table 3.2)	1 ml l^{-1}
Trace elements solution 7 (Table 3.3)	1 ml l^{-1}

The reactor containing 1 L of concentrated culture medium (except glycerol and $FeSO_4.7H_2O$) was autoclaved for 30 min. Sterile concentrated glycerol and $FeSO_4.7H_2O$ solutions accounting for 80 ml were aseptically added to the reactor, which was inoculated with 120 ml of an early exponential phase (12 h) culture of *C. pasteurianum* (strain M2 obtained in Chapter 5). The agitation was set at 150 rpm and the temperature was kept at 37 ºC by means of an external jacket for water circulation. The initial pH of the culture was set at 6.8 and pH control started when it reached the desired value by means of the addition of NaOH 2M. In order to avoid contamination, a sterile 0.2 µm filter was installed in the N_2 line before the reactor entrance. The N_2 flow supplied to the reactor was controlled by a mass flow controller (Aalborg). The gas was introduced at the bottom of the reactor and dispersed into the liquid through a circular sparger.

Three different experiments were conducted as it is presented in Table 7.2.

Table 7.2. - Experiments conducted to evaluate the effect of pH and N_2 sparging

Experiment	pH	N_2 flow
1	6.0	0.5 vvm
2	6.0	0.1 vvm during the lag phase[1]
3	5.0	0.1 vvm during the lag phase[2]

[1,2] The N_2 flow was cut off when cells started to grow.

Samples were taken at regular time intervals and soluble products were analysed as described in Section 3.2.2.

7.3 Results and discussion

The fermentation of glycerol by *C. pasteurianum* at pH 6.0 and different N_2 sparging conditions is shown in Figures 7.1 to 7.4. Figures 7.1 and 7.2 illustrate the solvents and acids production in the experiment conducted at pH 6.0 using 0.5 vvm N_2. Figures 7.3 and 7.4 correspond to the experiment at pH 6.0 in which 0.1 vvm N_2 was supplied during the lag phase.

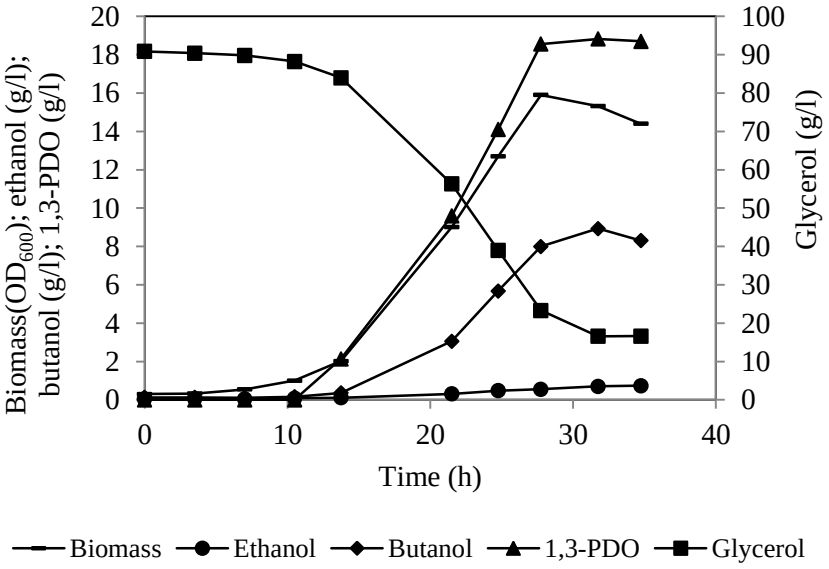
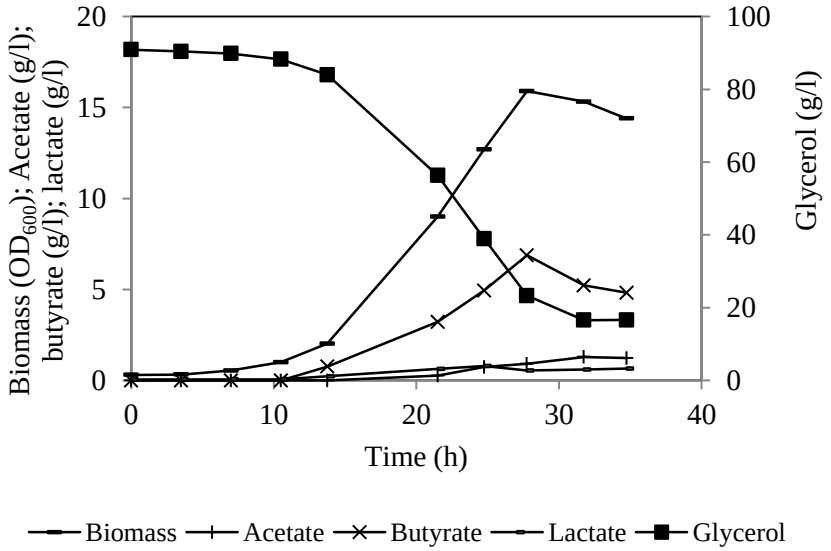


Figure 7.1. - Solvent production from pure glycerol by *C. pasteurianum* at pH 6.0 using 0.5 vvm N_2



Figur 7.2. - Acid production from pure glycerol by *C. pasteurianum* at pH 6.0 using 0.5 vvm N_2

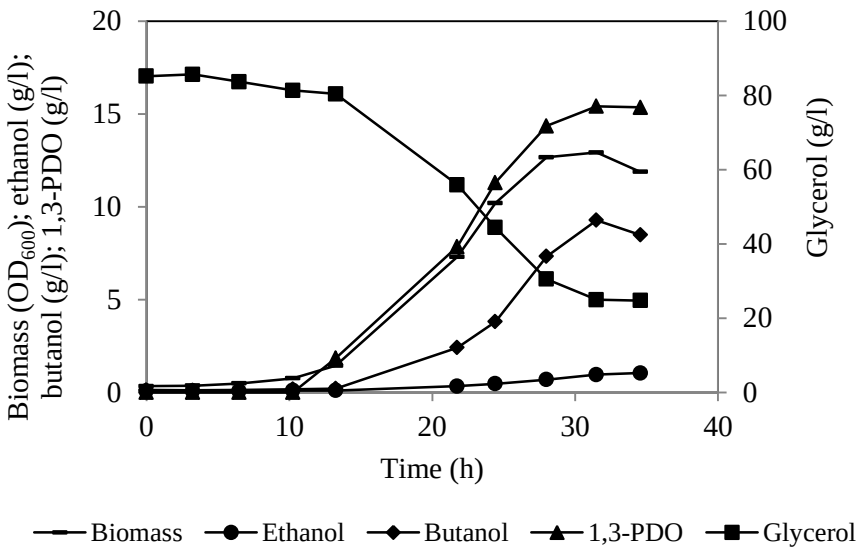


Figure 7.3. - Solvent production from pure glycerol by *C. pasteurianum* at pH 6.0 using 0.1 vvm N_2 during the lag phase

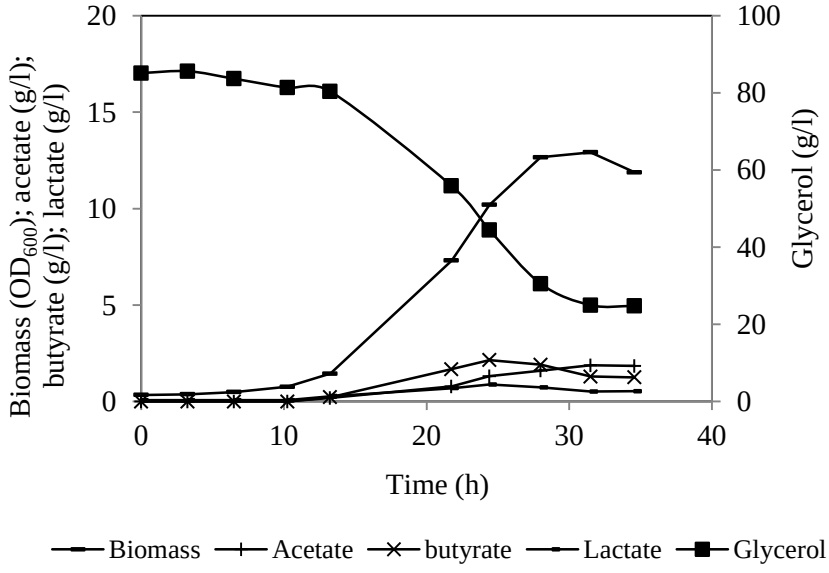


Figure 7.4. - Acid production from pure glycerol by *C. pasteurianum* at pH 6.0 using 0.1 vvm N_2 during the lag phase

The maximum glycerol consumption (74.93 g l^{-1}) was obtained in the fermentation controlled at pH 6.0 in which 0.5 vvm N_2 was supplied to the culture medium during the whole fermentation. However, the butanol titer was only 8.93 g l^{-1} and a high amount of 1,3-PDO (19.13 g l^{-1}) was produced. In the second experiment controlled at pH 6.0, in which 0.1 vvm N_2 was supplied to the culture

only until cells started to grow, glycerol consumption decreased to 61 g l^{-1}. Nevertheless, in this case the final butanol and 1,3-PDO titers were 9.28 g l^{-1} and 15.76 g l^{-1}, respectively. Therefore, this condition led to an increase in the butanol yield and resulted in the higher butanol volumetric productivity (0.29 g $l^{-1}h^{-1}$). As a comparison, Malaviya and collaborators (2011) reported a butanol volumetric productivity of 0.31 g $l^{-1}h^{-1}$ using a hyper butanol-producing mutant of *C. pasteurianum* DSM 525 in batch culture and 0.27 g $l^{-1}h^{-1}$ for the wild type strain.

An interesting point to highlight is the high cell density achived by the cultures. It has been reported that the maximum cell density achieved by butyric acid clostridia corresponds to an OD_{600} of 10-11 (Papoutsakis, 2008), which is in agreement with Malaviya and collaborators (2011) who reported a maximum OD_{600} of around 10 using *C. pasteurianum*. In the experiments presented in this chapter, a maximum OD_{600} of 15.9 was obtained in the fermentation conducted at pH 6.0 in which 0.5 vvm N_2 was used. Even in the second fermentation (pH 6.0 and 0.1 vvm N_2 during the lag phase), this value was 12.92.

Figure 7.5 and 7.6 present the results of the fermentation at pH 5.0 and 0.1 vvm N_2 during the lag phase.

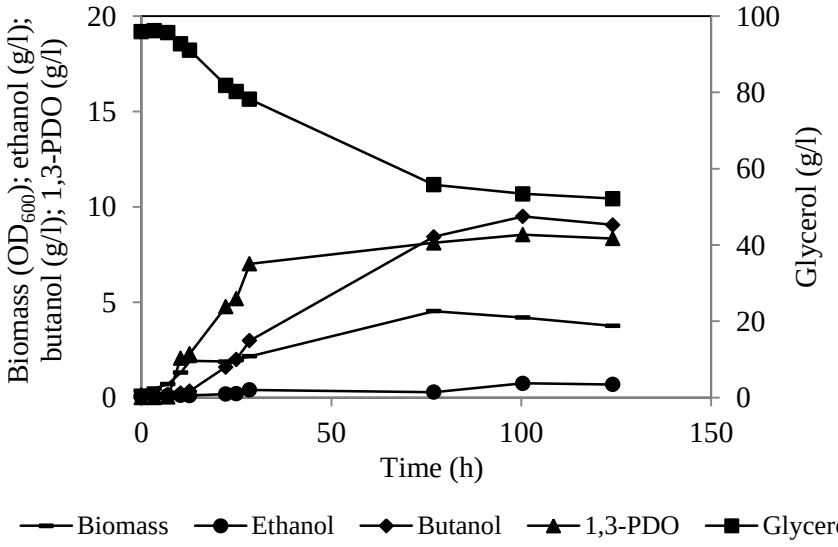


Figure 7.5. - Solvent production from pure glycerol by *C. pasteurianum* at pH 5.0 using 0.1 vvm N_2 during the lag phase

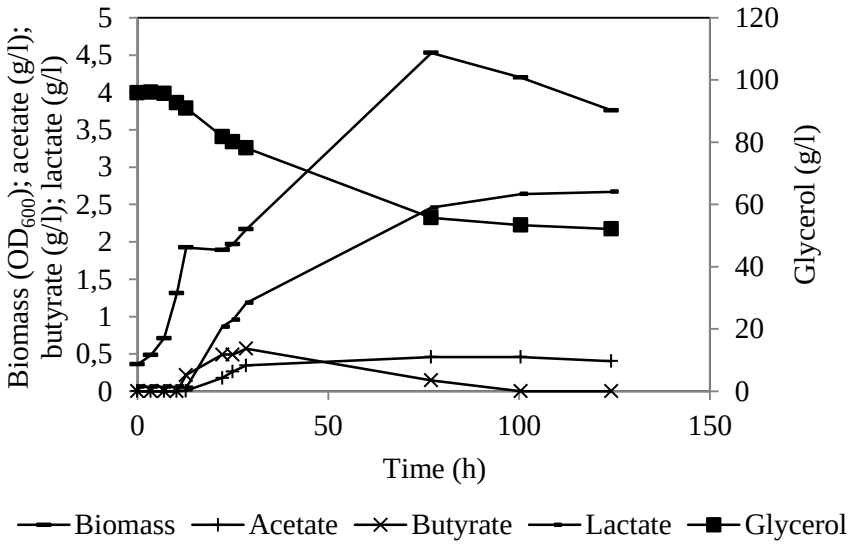


Figure 7.6. - Acid production from pure glycerol by *C. pasteurianum* at pH 5.0 using 0.1 vvm N_2 during the lag phase

In comparison with the experiments conducted at pH 6.0, the fermentation controlled at pH 5.0 showed a completely different behaviour. The glycerol consumption was only 43. 51 g l^{-1}, similar to the higher consumption obtained in serum bottles (45.62 ± 3.81). However, the final butanol titer was 9.5 g l^{-1}, which implies an even higher butanol yield. The concentration of 1,3-PDO reached 8.53 g l^{-1}. The fermentation was much longer than the ones controlled at pH 6.0, which resulted in a dramatic decrease in the butanol volumetric productivity (Table 7.3). Interestingly, although the butanol titer was similar to the obtained in fermentations controlled at pH 6.0, the cell concentration reached in this fermentation, measured as OD_{600}, was almost four times lower. Unlike the cultures controlled at pH 6.0, the re-assimilation of butyrate was complete although the maximum measured concentration was lower.

Table 7.3. - Butanol yield and volumetric productivity for glycerol fermentation by *C. pasteurianum* at constant pH values and N_2 sparging conditions

	Fermentation		
Parameter	**pH = 6.0 (0.5 vvm N_2)**	**pH = 6.0**	**pH =5.0**
Butanol yield (g g^{-1})	0.12	0.15	0.22
Butanol productivity (g $l^{-1}h^{-1}$)	0.28	0.29	0.09

Although the experiments conducted at pH 6.0 resulted in higher glycerol consumption and 1,3-PDO production in comparison with the fermentations in serum bottles (Chapter 6), the butanol production and yield were significantly lower. Based on data from the literature, this situation and the differences observed between experiments conducted in reactors at pH 6.0, with and without N_2 sparging, can be related with the specific production of hydrogen.

As it is shown in Figure 2.3 (Chapter 2), glycerol can enter the glycolitic pathway as the intermediate dihydroxyacetone-phosphate, which is then converted to pyruvate with electrons being transferred to nicotinamide-adenine dinucleotide (NADH). Pyruvate is oxidized by pyruvate: ferredoxin oxidoreductase (PFOR) to acetyl-CoA and CO_2, with electrons being transferred to ferredoxin (Fd). Hydrogen is produced by the hydrogenase enzyme, which catalyzes proton reduction using electrons from ferredoxin.

Under certain conditions, the fermentation broth could become supersaturated with hydrogen, limiting the formation of this compund and forcing the organism to channel electrons through NADH: ferredoxin oxidoreductase to reduce other intermediates such as acetyl-CoA to etanol as it has been reported for *C. thermocellum* (Doremus et al., 1985). In. *C. pasteurianum*, the situation is similar and an inhibition of hydrogen formation should necessarily result in higher yield of other reduced products such as 1,3-PDO, lactate and butanol to mantain the redox balance. In turn, it is likely that the ditribution of these products is regulated by complex mechanisms that respond to several factors such as environment conditions, nutrient availavility, physiological state of the cells, among others.

Basically, there are three variables that can influence the concentration of dissolved H_2 (and CO_2) in the fermentation broth; pressure, agitation and gas sparging.

The effect of the H_2 partial pressure on the ABE fermentation has been studied by several authors, some of which have been reviewed by Jones and Wood (1986). Generally, increasing the partial pressure of hydrogen in the headspace during the fermentation has shown to modulate the ABE fermentation resulting in a lower hydrogen production and higher butanol yield. Yerushalmi and collaborators (1985) reported that elevated H_2 partial pressure (in a range of 274 -1,479 kPa) in the acetone- butanol fermentation increased the butanol and ethanol yields on

glucose by an average of 18% and 13%, respectively; while the respective yields of acetone and endogenous produced H_2 decreased by an average of 40% and 30%, and almost no effect was observed on the growth of the culture.

Su and collaborators (1981), cited by Doremus et al. (1985), examined the effect of agitation, gas sparging, and head-space pressure on the ratio of ethanol to acetate production by *C. thermocellum*. The authors showed that the level of dissolved hydrogen gas was inversely proportional to the agitation rate and indeed dramatically affected solvent ratios in fermentations by *C. thermocellum*. Moreover, they found that *C. thermocellum* cultures when unstirred had three times more dissolved H_2 than stirred ones, indicating that H_2 transfer to the gas phase is rate limiting and that the broth can be supersaturated with this compund, simply by not stirring it. According with these results, the authors found that the etanol/acetate ratio increased from one in stirred cultures to three in unstirred ones, and that the hydrogen evolution rate was three times higher in the stirred, acetate-producing fermentations. The authors also found that H_2 production could be inhibited by increasing the H_2 partial pressure, and could be restored by sparging with nitrogen. Hussy et al. (2003), Kim et al. (2006), Mizuno et al. (2000) and Kraemer and Bagley (2006), working with undefined mixed cultures, reported that H_2 yield can be increased by sparging the birreactor with N_2. However, Kraemer and Bagley (2006) reported that H_2 and CO_2 were still supersaturated regardless of sparging.

Doremus and collaborators (1985) showed that in non-pressurized fermentations, the butanol productivity was inversely proportional to agitation. Considering the antecedents presented by Su and collaborators (1981), the butanol productivity was favored by conditions which result in high levels of dissolved hydrogen (Doremus et al., 1985). Opposite to the non-pressurized experiments, the authors reported that in fermentations conducted at 1 bar pressure, the maximum butanol productivity was directly proportional to agitation. However, it is worth to note that the butanol productivity does not depend exclusively on this variable.

In summary, conditions that yield an increase in the partial pressure of hydrogen in the culture medium such as high head-space pressure and low or nule agitation result in a decrease of hydrogen production and an increase of the production of butanol and etanol in solventogenic *Clostridium* spp.

It is worth to note that the results presented in Chapter 6 correspond to experiments in serum bottles, that were always overpressured and without any agitation. Therefore, it is reasonable to consider that the higher butanol yield obtained in that fermentations (in comparison with the ones presented in this chapter) was in part due to a higher dissolved H_2 concentration, which in turn inhibited hydrogen production forcing cells to redirect the carbon and electron flux towards the production of solvents. However, it is likely that the difference in the pH profile during the fermentation also played an important role. Similar arguments can be used to explain the differences in butanol yield between the cultures conducted at pH 6.0 with and without N_2 sparging. As Su and collaborators (1981) reported, N_2 sparging can restore H_2 production in the cases where hydrogenase is inhibited by a high H_2 partial pressure. In this context, the results herein presented suggest that although the experiments were conducted under agitation, this condition was not enough to avoid hydrogenase partial inhibition by dissolved H_2 concentration, and that probably the culture grown without N_2 sparging was supersaturated with H_2 thus leading to a higher butanol yield in comparison with the culture in which 0.5 vvm N_2 was used.

The pH has been recognized as an important variable in the ABE fermentation and the general consensus is that fermentations conducted at relatively high pH values produce acids rather than solvents, whereas in fermentations performed at relatively low pH values, the reverse is true (Maddox, 1989). However, there is a strong interaction between substrate concentration and pH, which differs depending on the strain used. Higher initial substrate concentrations encourage solvent production, even at neutral pH values (Maddox, 1989). Also, the supplementation of the culture medium with high concentrations of acetate and butyrate has allowed substantial solvent production at pH 7.0 using *C. beijerinkii* and a low susbstrate concentration (20 g l^{-1} glucose) (Holt et al., 1984).

Marchal and collaborators (1985), cited by Maddox (1989), working with a substrate derived from the Jerusalem artichoke, reported a marked influence of pH value. The authors showed that while a low pH value favoured solvent production over acid production, it also led to an increase in the total fermentation time. Ennis and Maddox (1987), cited by Maddox (1989), working with a substrate of whey permeate reconfirmed that at relatively low lactose concentrations (45 g l^{-1}),

a low pH favoured solvetogenesis, but growth and sugar utilization were poor. Conversely, at higher pH values, although growth and sugar utilization were much improved, solvent production was poor. On the other hand, George and Chen (1983) reported that cultures of *C. beijerinkii* maintained at pH 6.8 produced nearly as much butanol as those incubated without pH control using 60 g l^{-1} sacarose.

The results presented in this Chapter are in agreement with previously reported work. The good solvent production observed at pH 6.0 is likely a consequence of the high initial substrate concentration used (90 g l^{-1} glycerol). However, Biebl (2001) also reported good solvent production working with *C. pasteurianum* at pH 6.0 using 50 g l^{-1} glycerol. It is also possible that independently of its concentration, the highly reduced nature of glycerol encourage butanol production in *C. pasteurianum* at relatively high pH values, which does not happen with other substrates such as glucose or lactose. As an example, Husemann and Papoutsakis (1988) found that butanol production was initiated but not sustained in batch fermentations of controlled-pH 6.0 with an initial glucose concentration of 300 mM (54 g l^{-1}). Butanol production started when the butyrate concentration reached 100 mM. However, the final concentration of butanol never exceeded 2 mM, although the final concentration of butyrate was 160 mM. On the other hand, the fermentation conducted at pH 5.0 presented in this chapter favoured butanol production in terms of yield (Table 7.3), but as mentioned before lower glycerol consumption, biomass production and a longer fermentation time was observed.

Considering the maximum butyric acid concentration measured, its yield on glucose in the fermentation controlled at pH 6.0 and 0.5 vvm N_2 is around seven fold higher than in the fermentation controlled at pH 5.0. This situation, added to the higher butanol yield observed suggest that at low pH, genes responsible for acid production are downregulated, which results in lower ATP production; while genes involved in solvent production from butyryl-CoA are upregulated. A low ATP production can explain the lower biomass yield and longer fermentation time observed in the fermentation controlled at pH 5.0.

It is worth mentioning that in the experiments herein presented, indepently of the pH, the contribution of butiric acid to the production of solvents is minimum and most butanol should have been produced directly from acetyl- CoA through

butyryl-CoA. In the case of the experiments conducted at pH 5.0, although butyric acid is completely reassimilated, its production was vey low (< 0.6 g l^{-1}) as mentioned before. On the contrary, the production of butyric acid at pH 6.0 is considerable, however at this pH most acid is dissociated and therefore it cannot be assimilated since it cannot cross the cell membrane. This situation is reflected in the low decrease the concentration of butyric acid measured towards the end of the fermentation (Figures 7.2 and 7.4).

Another factor that probably contributed to the higher butanol yield observed in the fermentation conducted at pH 5.0 may be a low hydrogenase activity. It has been reported that hydrogenase activity measured in whole cells from acid-producing cultures maintained at pH 5.8 is about 2.2 times higher than that measured in solvent-producing cultures maintained at pH 4.5 (Andersch et al., 1989; Kim and Zeikus, 1985; cited by Jones and Woods, 1986). In a study carried out with *C. beijerinckii*, George and Chen (1983) also reported that extracts from solvent-producing cells exhibited lower levels of hydrogenase activity than those from acid-producing cells. In fact, as mentioned previously, a low hydrogenase activity forces the cell to redirect the carbon and electron fluxes towards the production of solvents.

Different conclusions about the lower hydrogenase activity observed has been reported. Kim and Zeikus (1985), cited by Jones and Woods (1986), investigated the effect of pH and fatty acids on *in vivo* hydrogenase activity. They found that under the assay conditions used neither pH nor fatty acid concentration affected the hydrogenase activity, and they concluded that the decrease in hydrogen production in the solventogenic phase was due to the regulation of hydrogenase production rather than inhibition of enzyme activity. Andersch and collaborators (1983), cited by Jones and Woods (1986), reported that hydrogenase activity in acid- and solvent-producing cells was similar under the same assay conditions. However, the hydrogenase activity in solvent producing cells could only be detected in the assay after a lag period of 10 to 15 min. The hydrogenase activity was optimal at a pH of 8.5 and no activity could be detected below pH 6.0. The authors concluded that the hydrogenase from solvent producing cells grown at pH 4.5 was present in an inactive form but was activated after a given lag period under the conditions used in the assay.

The lactic acid pathway is not operational under normal conditions, and it only appears to operate as a less efficient alternative to allow energy generation and the continued oxidation of NADH when the mechanisms for the disposal of protons and electrons by the generation of molecular hydrogen is blocked (Jones and Woods, 1986). Lactate production has been reported to occur when the activity of hydrogenase is inhibited by carbon monoxide (Kim et al., 1984; Jones and Woods, 1986). In the experiments controlled at pH 5.0, a higher lactate production was observed, which based on these antecedents could be related with the low hydrogenase activity mentioned before.

An interesting fact that can be observed in Figures 7.1, 7.3 and 7.5 is that most of the butanol is produced when cells are in the exponetial phase, as it has also been showed by Biebl (2001) and Malaviya et al. (2011). This situation supports the peviously proposed hypothesis about that most butanol is produced direcly from acetyl-CoA through butyril-CoA and not via acid production and subsequent reassimilation. In *C. acetobutylicum* and *C. bejerinkii*, eventhough solvent production can be observed in exponentially growing cells, significant solvent accumulation occurs in cells beyond the logarithmic growth phase (Rogers et al., 2006), associated with the reassimilation of acids. Moreover, it is known that the initiation of solvent production and sporulation are both regulated by the protein SpoOA (Alsaker et al., 2004; Harris et al., 2002; Ravagnani et al., 2000) as it was pointed out in Chapter 6. Therefore, this fact suggests that unlike other solventogenic *Clostridium* species, such as C. acetobutylicum and C. *beijerinkii*, sporulation and solvent production in *C. pasteurianum* are not coupled but independently regulated.

7.4 Conclusions

Both pH and N_2 sparging were shown to affect the glycerol fermentation by *C. pasteurianum*. Nitrogen sparging was found to improve glycerol consumption and change the product distribution probably by decreasing the dissolved gases concentration. In particular, it resulted in higher glycerol consumption and lower butanol yield, indicating that in the culture without N_2 sparging the concentration of dissolved H_2 was high enough to partially inhibit hydrogenase, even though

agitation was used. The pH effect observed is likely to be related with gene expression regulation. The control of pH value at 5.0 led to a higher butanol yield in comparison with pH 6.0. However, lower glycerol consumption and a longer fermentation was observed in comparison with the experiment performed at pH 6.0. The product yields obtained indicate that at low pH the genes related with acid production are probably downregulated, while those involved in solvent production are upregulated, thus resulting in a lower ATP generation and higher butanol yield. The product profiles of the cultures showed that the fermentation of glycerol by *C. pasteurianum* differs from the fermentation of sugars by other solventogenic clostridia in that most butanol is formed during exponential phase direcly from butyril-CoA instead of using the acid reassimilation pathway, and also suggest that the initiation of solvent producion and sporulation are independently regulated in this strain.

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Chapter 8

1,3-propanediol production from glycerol in EGSB reactors

1,3-Propanediol (1,3-PDO) was produced in Expanded Granular Sludge Blanket (EGSB) reactors fed with glycerol at 25 g l^{-1} at hydraulic retention times (HRT) between 3 and 24 h. Three parallel reactors were inoculated with anaerobic granular sludge (control reactor-R1), with heat-treated anaerobic granular sludge (R2) and with disrupted granular sludge (R3). A maximum yield of 0.52 mol of 1,3-PDO mol^{-1} glycerol was achieved in the reactor operated with non-treated granular sludge (control) at an HRT of 12 h, and a maximum 1,3-PDO productivity of 57 g l^{-1} d^{-1} was obtained also for the control reactor at 3 h HRT. Molecular biology tools were used to evaluate the bacterial community present in the biomass for each HRT studied and the inoculum. DGGE profiling of PCR-amplified 16S rRNA gene fragments showed that variations in the HRT had a critical impact in the dominant community of microorganisms. No appreciable differences in the bacterial population were observed between the reactors operated with heat–treated and disrupted granules at low HRTs. Production of H_2 was observed at the beginning of the operation, but no methane production was observed. This study proves the feasibility of 1,3-PDO production in anaerobic granular sludge reactors, which have the advantage of being operated under non-sterile conditions and represent a novel strategy to valorise glycerol generated as by-product in the biodiesel industry.

8.1 Introduction

The increasing demand for energy, sustained increase in the price of crude oil, and environmental concerns together with incentives for production of biofuels have been driving forces for the rapid growth of worldwide biodiesel production in the last years. As a consequence, a surplus of crude glycerol, a by-product of biodiesel production that represents 10 % w/w of the product has been generated thus creating a glut in the market (Johnson and Taconi, 2007). Furthermore, the disposal of these massive amounts of glycerol became a complex and expensive process, imposing a great pressure in this industry. Since the supply of oils to be converted to biodiesel is becoming increasingly competitive, the profitability of the biodiesel industry will depend on the ability to confer value to its by-products and therefore, the conversion of crude glycerol into other useful products is required (Choi 2008; Johnson and Taconi 2007).

The anaerobic biological conversion of glycerol is particularly interesting due to the highly reduced nature of this compound (Choi 2008). Its fermentation results in the generation of more reducing equivalents when it is converted to glycolytic intermediates as compared with glucose fermentation (Yazdani and Gonzalez, 2007). This excess of reducing equivalents must be oxidized which can be accomplished by the production of H_2 and/or via various NAD(P)H consuming pathways towards reduced or neutral end products (Heyndrickx et al., 1991). In fact, glycerol can be converted into several compounds such as citric acid, lactic acid, formic acid, acetic acid, butyric acid, propionic acid, succinic acid, dihydroxyacetone (DHA), 1,3-PDO, dichloro-2- propanol (DCP), acrolein, hydrogen, butanol, ethanol, among others (Choi 2008; Dharmadi et al., 2006; Yang et al., 2012).

In particular, 1,3-PDO is a versatile organic chemical used for the production of polyesters, polyethers and polyurethanes (Chuah et al., 1995, Kaur et al., 2012). This product is highly specific for glycerol fermentation and cannot be obtained from any other anaerobic conversion (Homann et al., 1990). Glycerol can be fermented into 1,3-PDO by species of *Klebsiella*, *Clostridium*, *Lactobacillus*, *Enterobacter* and *Citrobacter* (Barbirato et al., 1995; Boenigk et al., 1993; Johnson and Taconi 2007; Kaur et al., 2012; Leja et al., 2011;Yang et al., 2012). Specifically, this process has been demonstrated in *Lactobacillus brevis*,

Lactobacillus buchnerii, Bacillus welchii, Citrobacter freundii, Klebsiella pneumoniae, Klebsiella oxytoca, Clostridium pasteurianum, Clostridium butyricum and Eenterobacter agglomerans, (Chatzifragkou et al., 2011; Colin et al., 2000; da Silva et al., 2009; Gonzalez-Pajuelo et al., 2005a; Günzel et al., 1991; Homann et al., 1990; Menzel et al., 1997; Moon et al., 2010; Papanikolaou et al., 2004; Zeng et al., 1993; Zhang et al., 2006). Also, some studies using genetically modified *Clostridium acetobutylicum* have been reported (Gonzalez-Pajuelo et al., 2005b; Gonzalez-Pajuelo et al., 2006).

C. butyricum has been recognized as one of the best candidates for 1,3-PDO production. Among its several advantages, being a non-pathogenic microorganism and its ability to produce 1,3-PDO with yields close to the theoretical maximum are the most interesting ones (Gonzalez-Pajuelo et al., 2005a; Saint-Amans et al., 1994). Moreover, it is the only microorganism identified so far to use a coenzyme B12-independent glycerol dehydratase (Leja et al., 2011), which indeed has economical implications. Nevertheless, this strain has a relatively low tolerance to glycerol. It has been reported that *C. butyricum* is inhibited at 8% (w/v) glycerol (Biebl 1991). In this context, *C. pasteurianum* has the advantage of tolerating glycerol concentrations up to 17% (w/v) (Dabrock et al., 1992). On the other hand, it has also been reported that for *C. butyricum* CNCM 1211 the growth was completely inhibited at a 1,3-PDO concentration of 65 g l^{-1} (Colin et al., 2000).

As an alternative to pure cultures, 1,3-PDO can be produced using open mixed cultures to be operated under non-sterile conditions. These cultures, composed by a great microbial diversity, are able to degrade a variety of substrate mixtures.

Particularly interesting is the use of Expanded Granular Sludge Bed (EGSB) reactors, a system where microorganisms are naturally immobilized, allowing maintaining a high biomass concentration inside the reactors under low hydraulic retention times (HRT), which could potentially result in high volumetric productivities. Even though this kind of system has been widely used to treat wastewater, it has not been fully exploited for the production of added value compounds from industrial by-products such as glycerol. Typically, the degradation of sugars using this system at high retention times results in methane production, which indeed would have a detrimental effect in the production of 1,3-PDO. Moreover, it has been proposed that methanogenic archaea are placed as

a first layer surrounding the nucleus of the granules (McHugh et al., 2003) and therefore, methanogenic activity could potentially persist even at low retention times due to the impossibility of washing out these microorganisms from the reactor.

Several strategies have been used to minimize methane production by open mixed cultures, mainly in the context of hydrogen production, being heat and chemical inhibitors the most common ones. The basis of the heat treatment is to suppress non-spore forming hydrogenotrophic bacteria by applying heat (usually 100 ºC) for a certain period of time (Abreu et al., 2011). Examples of microorganisms able to survive after heat-treatment of granular sludge are those belonging to the *Clostridium* genus, including *C. butyricum*, which are hydrogen producing and spore-forming bacteria (Hallenbeck et al., 2009; Leja et al., 2011). The main problem with this strategy is that some microorganisms able to produce hydrogen but not heat-resistant spores could be eliminated in the process (Zhu and Béland, 2006).

Another possible strategy is the disruption of the granules. This strategy is based on the fact that methanogenic archaea have a lower specific growth velocity than 1,3-PDO producing bacteria such as *Clostridium* spp. and therefore, they will be washed out from the reactor at low hydraulic retention times.

In this work, the yield and productivity of 1,3-PDO production from glycerol and the microbial community is investigated for the first time in three parallel continuous high-rate EGSB reactors operating at hydraulic retention times between 3 and 24 h. A control reactor was inoculated with granular sludge without any treatment (R1 - control reactor), and heat-treated and disrupted granules were used as inoculum in reactors R2 and R3, respectively.

8.2 Material and methods

8.2.1 Inoculum source

Granular sludge was obtained from an up-flow anaerobic sludge blanket reactor used to treat brewery wastewater. The water treatment facility is located in Lisbon, Portugal.

8.2.2 Experimental procedure

Two pre-treatments were applied to the granular sludge: heat, which consisted in (i) autoclaving the granular sludge at 100 ºC for 15 minutes; and (ii) disruption of the granules using a 0.6x25 mm syringe. Granular sludge without treatment was used as control.

Continuous fermentations were conducted at 37 ºC in three EGSB reactors (working volume of 375 ml) operated in parallel. Each reactor was inoculated with 100 ml of sludge and fed with the semi-defined culture medium presented in Table 8.1.

Table 8.1. - Culture medium used for 1,3-PDO production in EGSB reactors

Compound	**Concentration**
Pure glycerol	25 g l^{-1}
NH_4Cl	3 g l^{-1}
KH_2PO_4	0.5 g l^{-1}
K_2HPO_4	0.5 g l^{-1}
$CaCl_2$.2H2O	0.02 g l^{-1}
$MgSO_4.7H_2O$	0.2 g l^{-1}
$NaHCO_3$	3 g l^{-1}
Yeast extract	1 g l^{-1}
Trace elements solution 6 (Table 3.2)	1 ml l^{-1}
Trace elements solution 7 (Table 3.3)	1 ml l^{-1}

Internal liquid recirculation was used in order to keep a suitable expansion of the granules inside the reactor (Figure 8.1). The initial pH of the medium was adjusted to 6.8. Different HRTs were applied starting with 24 h and afterwards changing it to 20 h, 12 h, 6 h and 3 h. Steady states were assessed by measuring the concentration of 1,3-PDO.

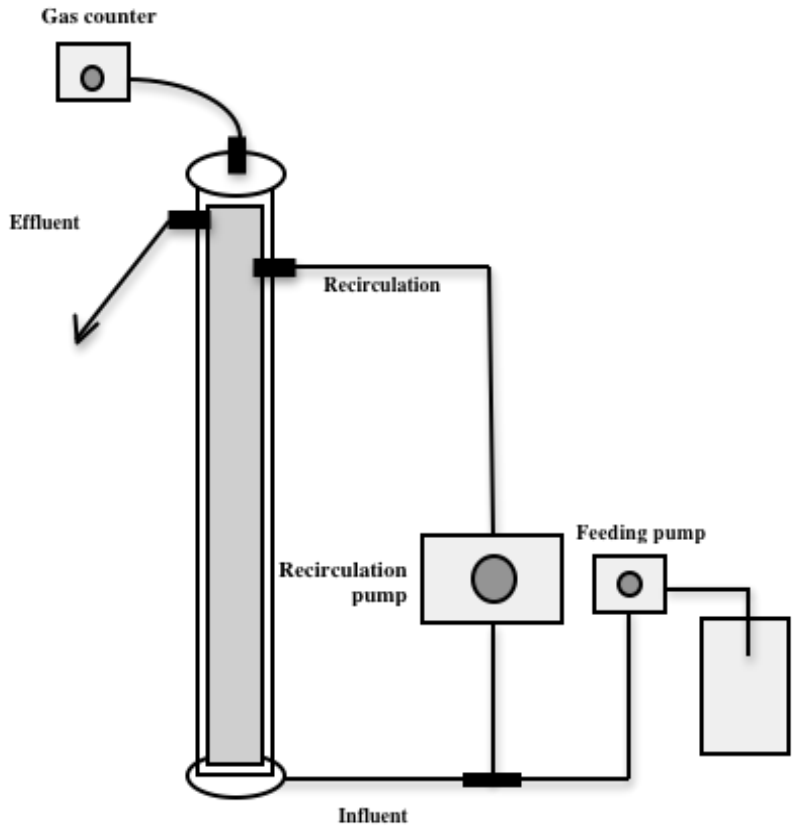

Figure 8.1. - EGSB reactor set up

8.2.3 Analytical methods

Acids, glycerol and 1,3-PDO were measured through high performance liquid chromatography (Jasco, Japan) equipped with UV and RI detectors. The column (Aminex cation-exchange HPX-*87H)* was eluted isocratically with H_2SO_4 0.01 N at 60 °C using a flow rate of 0.7 ml min^{-1}.

Biogas composition was analyzed by gas chromatography (Chrompack 9001) equipped with a thermal conductivity detector and two columns: Porapack Q (100-180 mesh) 2 m x 1/8'' x 2.0 mm SS column, and a MolSieve 5A (80-100 mesh) 1.0 m x 1/8'' x 2.0 mm SS. Argon was the carrier gas at a flow rate of 16 ml min^{-1}. The oven, injector and detector temperatures were 35, 110 and 110 °C, respectively.

8.2.4 Microbial community analysis

DNA Extraction and PCR-DGGE. For the inoculum and all HRTs studied, representative samples of biomass were collected and stored at -20 ºC until further treatment. Total genomic DNA was extracted using a FastDNA SPIN kit for soil (Qbiogene, Carlsbad, CA, USA) according to the manufacturer's instructions. The V6 to V8 region of bacterial 16S rRNA genes was amplified by PCR using the primers U968GC-f and L1401-r (Nübel et al., 1996). PCR products were separated by DGGE in a polyacrylamide gel (8%) containing a linear denaturing gradient ranging from 30% to 60% (100%-denaturing solution containing 7M urea and 40% formamide) using the DCode System (Bio-Rad Laboratories Inc, CA, USA). Electrophoresis was performed for 16 h at 85 V in 0.5X TAE buffer at 60 ºC. Gels were then stained with silver nitrate and scanned in an Epson Perfection V750 PRO (Epson, USA). Similarity indices (Si) were calculated from the densitometric curves of the scanned DGGE profiles using the Pearson product-moment correlation coefficient (Hane et al., 1993)

Cloning and sequencing. Bacterial 16S rRNA genes were amplified by PCR using the primers Bact27-f and 1492-r (Lane, 1991). The PCR products were purified with Nucleo Spin Extract II kit (Clontech Laboratories), ligated into pGEM-T vector (Promega, WI, USA) and introduced into competent *Escherichia coli* cells *E.cloni*®10G (Lucigen, WI, USA), according to the manufacturer's instructions. Cells of positive transformants were lysed and the 16S rRNA genes were amplified by PCR using the primers U968GC-f and L1401-r and screened in DGGE by comparison with the band-patterns of the sludge sample (template for cloning). Clones matching different bands in the total community profile were selected for further analysis. Selected clones were amplified using pGEM-T vector-targeted primers SP6/T7, purified with the Nucleo Spin Extract II kit (Clontech Laboratories, USA) and subjected to DNA sequence analysis. Sequencing reactions were performed at Eurofins MWG Operon (Germany). Similarity searches for the 16S rRNA gene sequences were performed using the BLAST search program within the GenBank database (http://www.ncbi.nlm.nih.gov/blast/) (Altschul et al., 1990).

8.3 Results and discussion

8.3.1 1,3- propanediol yield and productivity

Glycerol was mainly converted to 1,3-PDO in all the reactors. Acetate and butyrate were present but in low concentrations (<1 g l^{-1}), except in the reactor operated with heat-treated granular sludge when set at an HRT of 12h (up to 4 g l^{-1}) and the reactor operated with non-treated granular sludge at an HRT of 6h (1.8 g l^{-1}). pH was kept in the range 5-6 for the retention times of 24-12 h and decreased to the range 4-5 for the lower hydraulic retention times (6-3 h). Temudo et al. (2008) worked with CSTR using 4 g l^{-1} glycerol at an HRT of 8 h, pH 8 and found that ethanol and formate were the main products obtained. However, at increasing substrate concentrations, the yields of ethanol and formate decreased, while 1,3-PDO and acetate increased. Figure 8.2 illustrates the time course of glycerol and 1,3-PDO concentration for the three reactors.

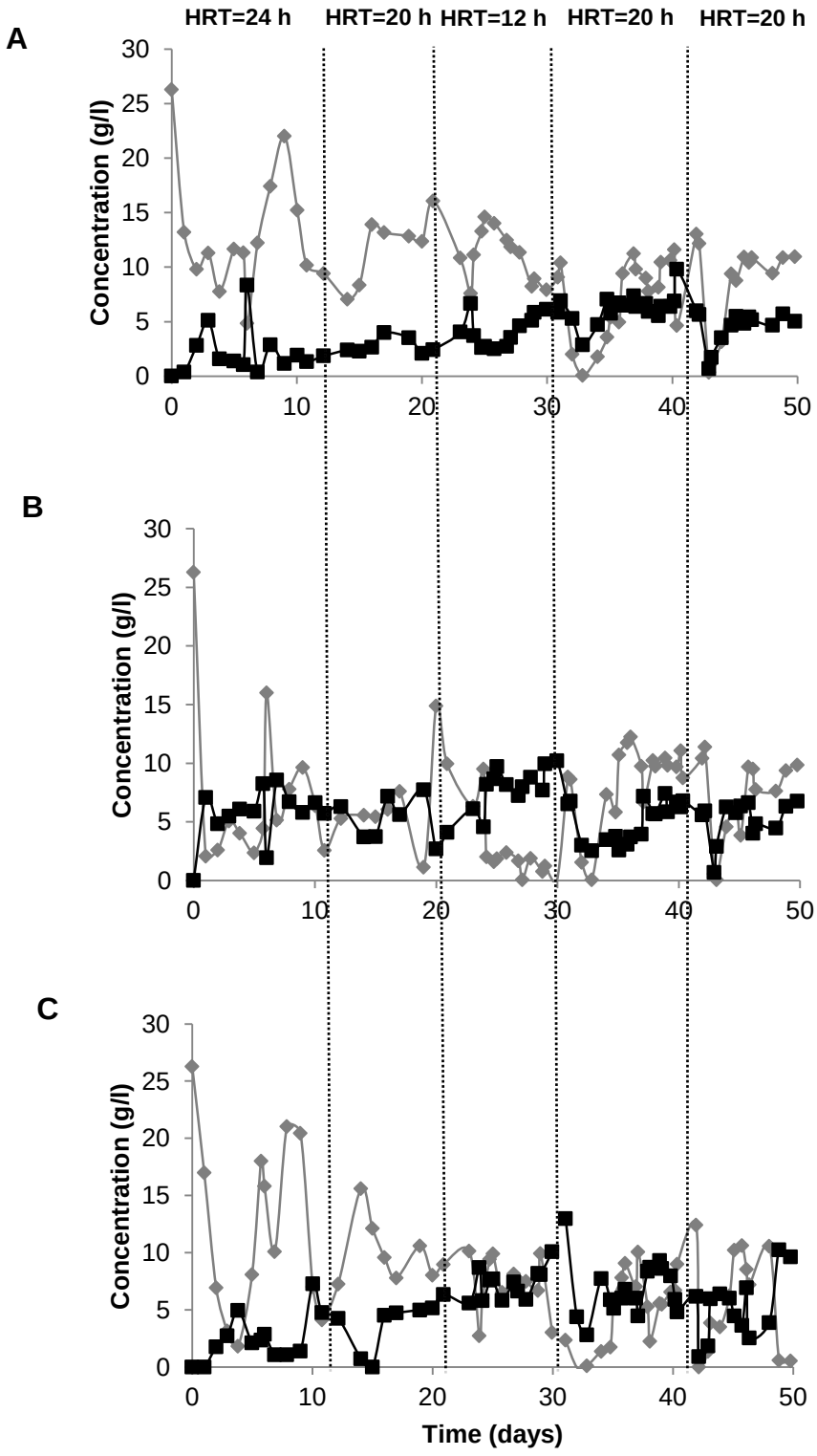

Figure 8.2. - Effect of HRT on the production of 1,3-PDO in the reactors operated with (A) disrupted granules; (B) heat-treated granules; and (C) non-treated granules. Black squares represent 1,3-PDO and gray diamonds represent glycerol concentrations

It is clear that 1,3-PDO concentration was more stable in the reactors inoculated with heat-treated granules and with disrupted granules, that in the control reactor.

Table 8.2 presents the 1,3-PDO yields and productivities for the three reactors and for all the hydraulic retention times applied.

Table 8.2. - 1,3-PDO yield and volumetric productivity

	1,3-PDO Yield (mol 1,3-PDO. mol^{-1} glycerol)			**1,3-PDO productivity (g 1,3-PDO. l^{-1}.d^{-1})**		
HRT (h)	Disrupted	Heat treated	Control	Disrupted	Heat treated	Control
24	0.21±0.14	0.39±0.07	0.31±0.09	1.5±0.33	6.7±1.1	3.2±2.1
20	0.29±0.08	0.37±0.05	0.36±0.05	3.6±1.1	7.0±2.2	6.2±0.90
12	0.39±0.08	0.42±0.05	0.52±0.08	11.7±1.3	16.6±2.7	16.2±4.2
6	0.46±0.08	0.47±0.05	0.44±0.07	26.9±4.2	25.8±2.7	30.9±4.8
3	0.39±0.04	0.37±0.08	0.40±0.07	39.5±5.2	45.1±7.8	56.9±16.8

In general, the 1,3-PDO yield was not influenced by the pre-treatment applied to the inoculum. Only for a HRT of 24 h a statistically different yield was observed between the reactors inoculated with disrupted and the heat-treated granules ($p=0.005$), and for a HRT of 12 h between the control reactor, which produced the highest 1,3-PDO yield, and the reactors inoculated with disrupted and heat-treated granules ($p= 0.01$ and $p=0.006$, respectively).

The HRT positively influenced the 1,3-PDO yield that significantly increased from 24 to 6 h. However, for a HRT of 3 h, a decrease in this parameter was observed.

The highest yields of 1,3-PDO from glycerol have been reported for pure cultures of *C. butyricum* by Saint-Amans et al. (1994) and Gonzalez-Pajuelo et al. (2005) corresponding to 0.56 g g^{-1} and 0.65 mol mol^{-1}, respectively; and for mixed cultures from wastewater (Selembo et al., 2009) corresponding to 0.69 mol mol^{-1}. The maximum theoretical yield for 1,3-PDO in co-production with acetate is 0.70 mol 1,3-PDO mol^{-1} glycerol (Beauprez et al., 2010; Biebl et al., 1999).

In this work, a maximum yield of 0.52 mol 1,3-PDO mol^{-1} glycerol was achieved. The results showed the same metabolic profile as the one described by Gonzalez-Pajuelo et al. (2005), in which 1, 3-PDO production is significantly lower when H_2 is being produced. Also, the maximum 1,3-PDO yield reported by Saint-Amans et al. (1994) was obtained without any H_2 production.

Concerning the volumetric productivity of 1,3-PDO, a maximum value was obtained for the control reactor at a HRT of 3 h, which was found to be significantly higher than the one obtained in the reactor inoculated with disrupted granules ($p=0.03$), but not significantly higher than the heat-treated granular based reactor. In general, for the control reactor, the 1,3-PDO volumetric productivity was higher than for the reactor inoculated with disrupted granules ($p=0.02$, 0.008, 0.008, 0.05, and 0.03, for HRT of 24, 20, 12, 6 and 3 hours, respectively). The differences in productivity between the reactor inoculated with heat-treated granules and the control reactor were only significant for HRT of 24 h ($p=0.0003$) and a HRT of 6 h ($p=0.02$). The reactor inoculated with heat-treated granules showed significantly higher productivities than the reactor inoculated with disrupted granules for HRT of 24, 20 and 12 h ($p<0.0001$, $p=0.04$ and $p=0.0008$, respectively).

The values of 1,3-PDO productivites herein obtained can be compared with literature values obtained with pure cultures (Table 8.3).

Table 8.3. - Data of yield and productivity of 1,3-PDO from glycerol obtained in this work and from literature

Microorganism	D (h^{-1})	Glycerol feeding ($g\ l^{-1}$)	1,3-PDO yield ($mol\ mol^{-1}$)	1,3-PDO Productivity ($g\ l^{-1}\ d^{-1}$)	Reference	Culture system
Undefined mixed culture (granular sludge)	0.04	25	0.31±0.09	3.2±2.1	This work	EGSB reactor
	0.05	25	0.36±0.05	6.2±0.90		
	0.08	25	0.52±0.08	16.2±4.2		
	0.17	25	0.44±0.07	30.9±4.8		
	0.33	25	0.40±0.07	56.9±16.8		
Undefined mixed culture (heat-treated granular sludge)	0.04	25	0.39±0.07	6.7±1.1	This work	EGSB reactor
	0.05	25	0.37±0.05	7.0±2.2		
	0.08	25	0.42±0.05	16.6±2.7		
	0.17	25	0.47±0.05	25.8±2.7		
	0.33	25	0.37±0.08	45.1±7.8		
Undefined mixed culture (disrupted granular sludge)	0.04	25	0.21±0.14	1.5±0.33	This work	EGSB reactor
	0.05	25	0.29±0.08	3.6±1.1		
	0.08	25	0.39±0.08	11.7±1.3		
	0.17	25	0.46±0.08	26.9±4.2		
	0.33	25	0.39±0.04	39.5±5.2		
Clostridium butyricum VPI 3266	0.4	30	0.6	132	(Gonzalez-Pajuelo et al., 2005a)	Single stage continuous culture
	0.3	60	0.65	247.2		
Klebsiella pneumoniae DSM 2026	0.1-0.25	na	na	117.6-211.2	(Menzel et al., 1997)	Anaerobic fixed bed reactor with effluent recycle
Clostridium butyricum F2b	0.11 – 0.04	90	0.59 (global)	31.92 (global)	(Papanikolaou et al., 2008)	Two-stage continuous culture (CSTR)

Table 8.3. - Continued

Microorganism	D (h^{-1})	Glycerol feeding ($g\ l^{-1}$)	1,3-PDO yield ($mol\ mol^{-1}$)	1,3-PDO Productivity ($g\ l^{-1}\ d^{-1}$)	Reference	Culture system
Citrobacter Freundii DSM 30040	0.5	36.8	0.57	196.8	(Pflugmacher and Gottschalk, 1994)	Anaerobic fixed bed reactor with effluent recycle
Clostridium acetobutylicum DG1 (pSPD5)	0.05	60	0.64	72	(Gonzalez Pajuelo et al., 2005b)	Single stage continuous cultureCSTR
Clostridium butyricum DSM 5431	0.31	30.39	0.65	74.4	(Reimann et al., 1998)	Continuous culture with cell recycling
Clostridium butyricum DSM 5431	0.31	30.21	0.57	100.8	(Reimann et al., 1998)	Continuous culture with cell recycling
	0.49	30.76	0.60	168		
	0.70	34.07	0.59	228		
	0.97	34.99	0.65	172.8		
	0.20	57.10	0.60	100.8		
	0.30	57.65	0.58	187.2		
	0.50	55.25	0.63	319.2		
	0.72	55.62	0.63	393.6		
	0.99	57.19	0.61	266.4		
	1.00	56.17	0.61	606.1		

8.3.2 Gas composition

Biogas composition revealed that methane was not produced at any of the HRT tested and H_2 was only detected at an HRT of 24 h in the reactors containing non-treated and heat-treated granular sludge. This means that the operating conditions were unfavourable to methanogenic archaea. This result is in agreement with the work reported by Selembo et al. (2009) that did not observe methane production working in batch with heat-treated inocula using glycerol as carbon source. Temudo et al. (2008) reported almost negligible production of H_2 (0.4 % of the products) working in CSTR using an HRT of 8 h, pH 8 and glycerol as the carbon source. These results point to a shift in the overall metabolism that can be explained by the selection process that occurs through the different HRTs. It is worth to note that hydrogen-consuming microorganisms, such as acetogenic bacteria, can survive to heat treatment (Oh et al., 2003).

8.3.3 Microbial community profiles

The results obtained by DGGE clearly show that the bacterial community changed significantly through the different HRTs applied (Figure 8.3). The profiles of the heat-treated, disrupted and non-treated biomass collected at the lowest HRT tested, i.e. 3 h, exhibited a similarity to the correspondent inocula of only 29.5% (H3h/InH), 21.4% (D3h/InD) and 18.2% (C3h/InC), respectively (Figure 3b).

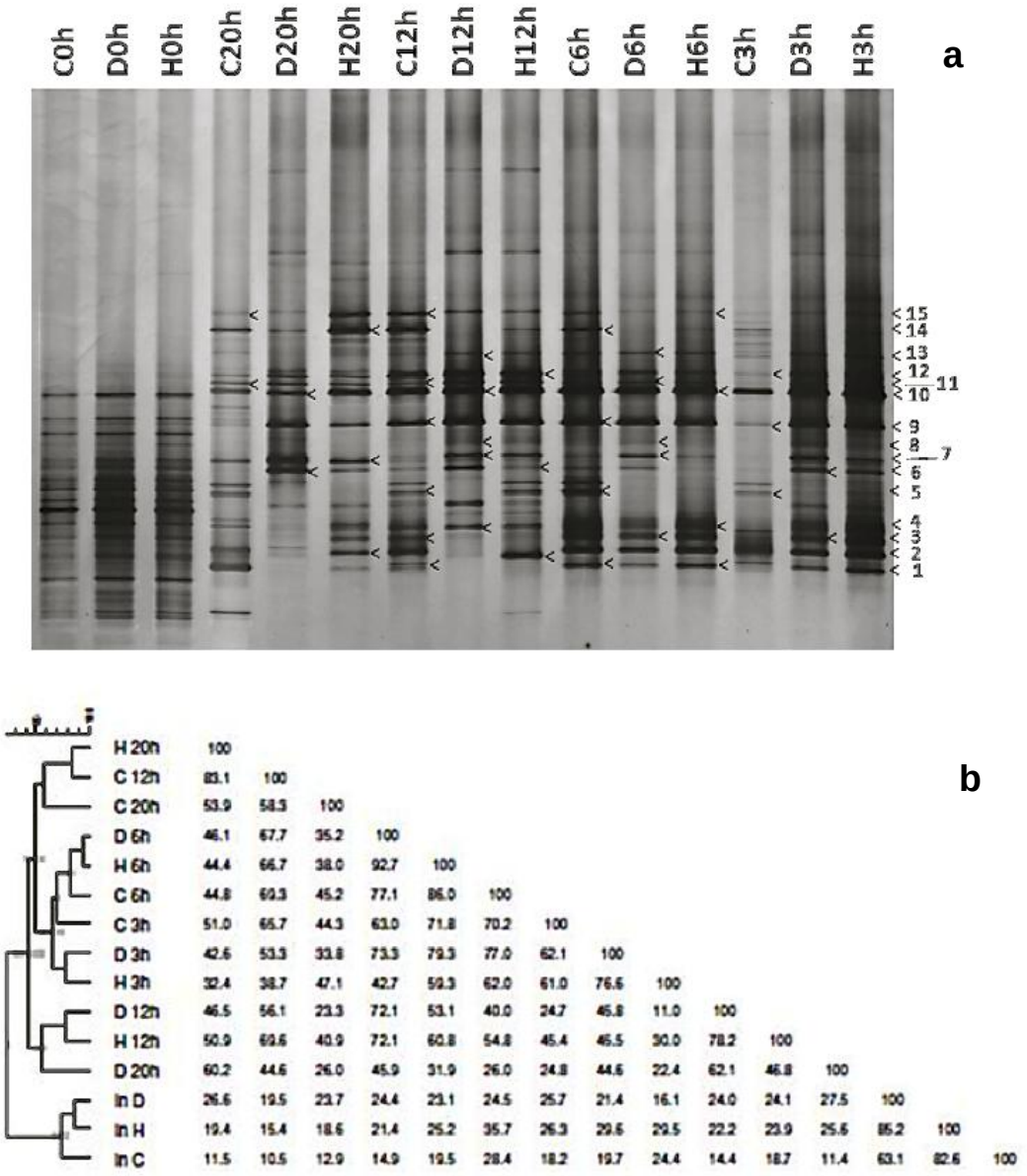

Figure 8.3. - Bacterial DGGE profile (a) of the granular sludge at times 0, 20, 12, 6 and 3 h for the reactors operated with non-treated biomass (C), disrupted biomass (D) and heat-treated biomass (H) and corresponding similarity indices (in %) dendogram (UPGA clustering) and matrix (b). Numbers 1 to 15 marks the band position of the sequenced 16S RNA genes

Comparing samples from the different reactors it is perceptible that the bacterial community evolved differently according to the initial treatment. Nevertheless, no significant differences were found at the lowest HRT tested, at least between the reactors operated with heat-treated and disrupted granular sludge (H3h/D3h -

76.6%). Bacterial 16S rRNA gene sequences retrieved from the sludge samples showed high levels of similarity (>97%) to those of members of the genus *Lactobacillus* (bands 1,2,3,4, and 10), *Vagococcus* (11, 12 and 13), *Clostridium* (bands 14 and 15), *Enterococcus* (bands 6 and 7), *Klebsiella* (band 8), *Enterobacter* (band 9) and to uncultured bacteria from the *Clostridiaceae* family (band 5) (Table 3)

Table 8.4. - Affiliations of the cloned 16S rRNA gene sequences

Band ID	Closest relatives (% sequence similarity)
1	*Lactobacillus delbrueckii* (99%)
2	Uncultured bacterium clone VHW_F_D1 (99%), *Lactobacillus delbrueckii* (99%)
3	*Lactobacillus parabuchneri* (99%)
4	Uncultured bacterium clone MY33 (99%), *Lactobacillus delbrueckii* (99%)
5	Uncultured bacterium clone Ta_1_28 (98%), *Clostridium tyrobutyricum* (95%)
6	Uncultured bacterium clone inf86 (99%), *Enterococcus canintestis* (99%)
7	Uncultured bacterium clone inf86 (99%), *Enterococcus canintestis* (99%)
8	*Enterobacteriaceae* bacterium A52 (99%), *Klebsiella* sp. Gx17 (97%)
9	*Enterobacteriaceae* bacterium A52 (99%), *Enterobacter* sp. LB9 (98%)
10	Uncultured bacterium clone VHW_F_D1 (99%), *Lactobacillus parabuchneri* (99%)
11	Uncultured bacterium clone F2V (99%), *Vagococcus salmoninarum* (99%)
12	Uncultured bacterium clone RB-3A5 (98%), *Vagococcus carniphitus* (97%)
13	Uncultured bacterium clone RB-3A5 (98%), *Vagococcus salmoninarum* (99%)
14	*Clostridium butyricum* (NCIMB8082) (99%)
15	*Clostridium butyricum* strain AB33 (99%)

Even though members of all these genera have been reported as able to metabolize glycerol, the production of 1,3-PDO has not been proved for some of the species herein identified. As an example, *Lactobacillus delbrueckii* has been

shown to produce considerable amounts of lactic acid from glycerol and minor quantities of acetic acid (Choubisa et al., 2012).

Among the microorganisms herein detected known for producing 1,3-PDO, *C. butyricum* NCIMB8082 (99%), *C. butyricum* strain AB33 (99%), and *Lactobacillus* were the most representative. *Lactobacillus panis* PM1 was reported to produce 1,3-PDO from bioethanol thin stillage and *L. coryniformis* and *L.* parabuchneri to produce 1,3- and 1,2-PDO from glycerol and lactate, respectively (Khan et al., 2013; Sekwati-Monang et al., 2012). Regarding Enterobacteriaceae, it has been pointed out that anaerobic growth in glycerol and formation of 1,3-PDO is used as a characteristic mark for the identification of the genus Citrobacter, but it can also be found in strains of Klebsiella (Toraya et al., 1980). Furthermore, it has been described that some lactic acid bacteria produce 1,3-PDO from glycerol if both a growth and energy substrate is present in the medium (Schütz and Radler, 1984).

Finally, it is important to mention that the reactor that exhibited the highest 1,3-PDO volumetric productivity, operated with non-treated biomass at an HRT of 3h (sample C3h), showed a bacterial profile considerably different from the other two (samples H3h and D3h) (Figure 3). Bands corresponding to microorganisms related to *Enterococcus* (band 6 and 7), *Klebsiella* (band 8) and *Enterobacter* (bands 9) had a lower relative dominance in sample C3h, some being undetectable. Two rybotypes closely affiliated to *Lactobacillus*-like organisms (bands 2 and 10) seems to dominate in this bacterial community.

8.4 Conclusions

Continuous production of 1,3-PDO from glycerol using granular sludge in EGSB reactors is described for the first time. The production yield (0,52 mol 1,3-PDO mol^{-1} glycerol) is not as high as the values reported for pure cultures, however the advantage herein is that fermentations are conducted in continuous non-sterile conditions which has the potential of significantly decreasing the operational costs. Also, the conditions used were found to suppress the metabolism of the methanogenic archaea, thus resulting in the absence of methane production. Furthermore, the results obtained suggest that at low retention times the pre-

treatment applied to the biomass has no significant effect, being anaerobic granular sludge a suitable catalyst for the continuous production of 1,3 PDO from glycerol.

8.5 References

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Chapter 9

General conclusions and recommendations for future work

9.1 General conclusions

Clostridium pasteurianum DSM 525 shows a great potential for the production of butanol and 1,3-PDO. However, the inhibition by butanol limits the concentration of this compound in the culture medium. Through the experiments conducted in this work, it was established that the wild-type strain produces maximally 9 to 10 g l^{-1} of butanol, which is in agreement with the values reported in literature.

C. pasteurianum DSM 525 showed to be affected by strain degeneration as it has been reported for other solventogenic species. However, the isolation of a degeneration-resistant strain was possible through repeated subculture and selection.

The competitive nature of butanol and 1,3-PDO pathways in *C. pasteurianum* was evident through this PhD work. The use of high glycerol concentrations favours the production of butanol and it was demonstrated that the culture medium composition and operational variables greatly influences the product disribution. In particular, iron concentration, butyrate supplementation, pH and N_2 sparging were found to be key variables, being possible to modulate the fermentation to favour the production of either butanol or 1,3-PDO. Furthermore, it was found that the use of an inoculum composed of early exponential-phase cells (12 h) results in a notorious increase in glycerol consumption and butanol production. These two parameters decrease as the incoculum age increases.

The random mutagenesis experiments conducted in solid medium showed that the technique used is an efficient tool that allowed increasing both the butanol production and tolerance of the strain. The maximum butanol titer obtained in liquid medium (12.4 g l^{-1}) is in agreement with the results obtained in solid medium, thus suggesting that it is not possible to obtain higher butanol concentrations with the isolated strain without further genetic manipulations.

Experiments in pH-controlled reactors resulted in almost twice the glycerol consumption in comparison with the results obtained in serum bottles. Even though higher concentrations of 1,3-PDO were obtained, the yield of butanol decreased in comparison with the values obtained in serum bottles, thus confirming the importance of pH and that conditions that lead to a high H_2 partial pressure are required to obtain higher butanol yields. It was demonstrated that a

low pH favours the production of butanol. However, a lower volumetric productivity was obtained under this condition due to a notorious increase in the fermentation time.

An interesting feature of the fermentation of glycerol by *C. pasteurianum* that differentiates it from *C. acetobutylicum* and *C. beijerinkii* is that most butanol is produced during the exponential growth phase, suggesting that the regulation of sporulation and solvent production are not coupled. Furthermore, the results obtained showed that acid reassimilation does not contribute significantly to the production of butanol by the strain under the conditions used.

On the other hand, continuous production of 1,3-PDO from glycerol using granular sludge in EGSB reactors was herein described for the first time. Yields closer to values reported for pure strains were achieved, what makes this fermentation system an interesting alternative to value the crude glycerol generated in the biodiesel industry. Moreover, it was demonstrated that the use of glycerol and the hydraulic retention time are good selection pressures, and therefore a biomass pre-treatment is not required for the production of 1,3-PDO.

9.2 Recommendations for future work

Based on the results obtained in this work, it is clear that inhibition by butanol is still the main problem to overcome in *C. pasteurianum*. However, the butanol yield should be also a parameter to improve.

Once the optimal culture conditions are established, further optimization of the process should be focussed on strain improvement by genetic manipulation.

One alternative is to apply the random mutagenesis technique, as described herein, for successive rounds of mutagenesis-selection. Mutagenic agents other than ENU can be evaluated. In particular, Nitrosoguanidine (NG) has been reported to be particularly effective in *Clostridium* spp.

A more rational approach would be to take advantage of the genome sequence and annotation of *C. pasteurianum* DSM 525. In this sense, as it has been done with other solventogenic *Clostridium* spp., such as *C. acetobutylicum* and *C. beijerinkii*, the first step should be the development of a genome-scale model that

described the *C. pasteurianum* metabolism. This model could be further refined, for example by analysis of a wide-genome dynamic transcriptome in batch culture. Once this objective is accomplished, an *in silico* analysis using Flux Balance Analysis (or other approaches) and available bioinformatic tools, such as COBRA or Optlux, could be used to identify genetic modifications that can lead to a superior genotype.

A priori, it is suggested to explore the possibility of knocking out those genes involved in acid production. In principle, the requirements of ATP of the cell are satisfied through glycolisis and therefore, the production of acetic, butyric and lactic acids could be eliminated, however the redox balance should be taken into account. Furthermore, the simultaneous knock out of genes involved in the production of butyric acid and acetic acid in *C. acetobutylicum* has been recently reported in literature (Jang et al., 2012). These modifications led to higher butanol titers, beyond the previously considered tolerance limit of the strain. The production of hydrogen should be also a main target since hydrogenase inhibition has been recognized as a useful way to redirect the electron flow towards the production of butanol and thus increasing its yield.

It is also recommended to study the relationship between sporulation and solvent production. In this thesis, preliminary experiments using the directed mutagenesis tool Clostron were conducted with the goal of knocking out the *spoOA* gene. However, the DNA restriction system of *C. pasteurianum* hindered a successful tranformation. It is strongly recommended to further explore this alternative as a tool for the genetic modification of the strain. Research on the restriction-modification systems present in the strain should be conducted to enable the use of this valuable tool and to exploit the genome sequence and annotation of the strain.

Regarding the production of 1,3-PDO by open mixed cultures in EGSB, the culture medium composition could be further optimized. Moreover, it would be interesting to adapt the biomass to higher glycerol concentrations.

9.3 References

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